



The Contribution of Cervicovaginal Microbiota and Hormonal Contraceptives to Genital Inflammation and HIV Acquisition Risk

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The Contribution of Cervicovaginal Microbiota and Hormonal Contraceptives to Genital Inflammation and HIV Acquisition Risk

A dissertation presented

by

Melis Nuray Anahtar

to

The Division of Medical Sciences

in partial fulfillment of the requirements

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The Contribution of Cervicovaginal Microbiota and Hormonal Contraceptives to Genital Inflammation and HIV Acquisition Risk

Abstract

The HIV epidemic persists in many parts of the world, with the majority of new infections occurring through the female genital tract (FGT). The permissiveness of the genital mucosa to HIV is modulated by the integrity of the epithelial barrier, the presence of pro-inflammatory cytokines, and the frequency of CCR5+CD4+ T cell targets. Here we focus on two biological perturbations: endogenous alterations in cervicovaginal bacteria and exogenous injectable progestin-only contraceptives (IPCs). We examine their effects on the genital mucosal environment and their link to HIV susceptibility in a cohort of young South African women.

We first sought to determine how genital microbiota modulate host inflammatory responses. The existing paradigm is that vaginal monocolonization by *Lactobacillus* is normal, and encroachment by other bacteria is pathologic. By characterizing cervicovaginal bacterial communities in 94 South African women using 16S *rRNA* and shotgun sequencing, we found that the majority of participants had low *Lactobacillus* abundance and high ecological diversity. One diverse *Prevotella*-containing community type strongly correlated with increased concentrations of multiple genital pro-inflammatory cytokines *in vivo*. We found that these cytokines were produced by epithelial cells and antigen presenting cells via different bacterial sensing mechanisms. Our results identify specific bacterial species that alter the inflammatory state of the FGT and may more broadly impact reproductive health in women.

We also investigated the immunological effects of IPCs, the most common form of birth control in sub-Saharan Africa. Although highly effective as a contraceptive, IPCs are

controversially associated with increased HIV susceptibility by an unclear mechanism. We found that IPC users had a 5.5-fold higher risk of acquiring HIV than women not using family planning ($p=0.0031$, 95% CI: 1.733 – 16.80). Phenotypic cellular analysis revealed that IPC users also had a significantly higher frequency of activated HIV target cells in the cervix. Since the availability of target cells in the genital mucosa enables early viral replication, recruitment or retention of these cells by IPCs may explain the observed increased HIV acquisition rates. Furthermore, IPC use was not associated with differences in genital cytokine levels, indicating that cervicovaginal bacteria and exogenous progesterone increase HIV susceptibility by unique pathways.

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This dissertation is dedicated to the FRESH study participants.
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Chapter 1: Introduction

Epidemiology of HIV

The epidemiology of the HIV epidemic has changed dramatically since the first cases of Acquired Immune Deficiency Syndrome (AIDS) were reported in 1981. At that time, clinicians in New York City and San Francisco noticed a disturbing increase in previously healthy homosexual men presenting with advanced opportunistic infections. This mysterious disease with no identifiable cause was initially called “Gay-related immunodeficiency” (GRID) and “the 4H disease,” as it presented primarily in Haitians, homosexuals, hemophiliacs, and heroin users. Eventually, it was renamed AIDS (2). Today in 2015, 35 million men, women, and children around the world are living with HIV. While men who have sex with men continue to be 19 times more likely to be HIV-positive than the general population (3), 15% of people living with HIV are women between the ages of 15-24 and 80% of those young women live in sub-Saharan Africa (**Figure 1.1**) (4).

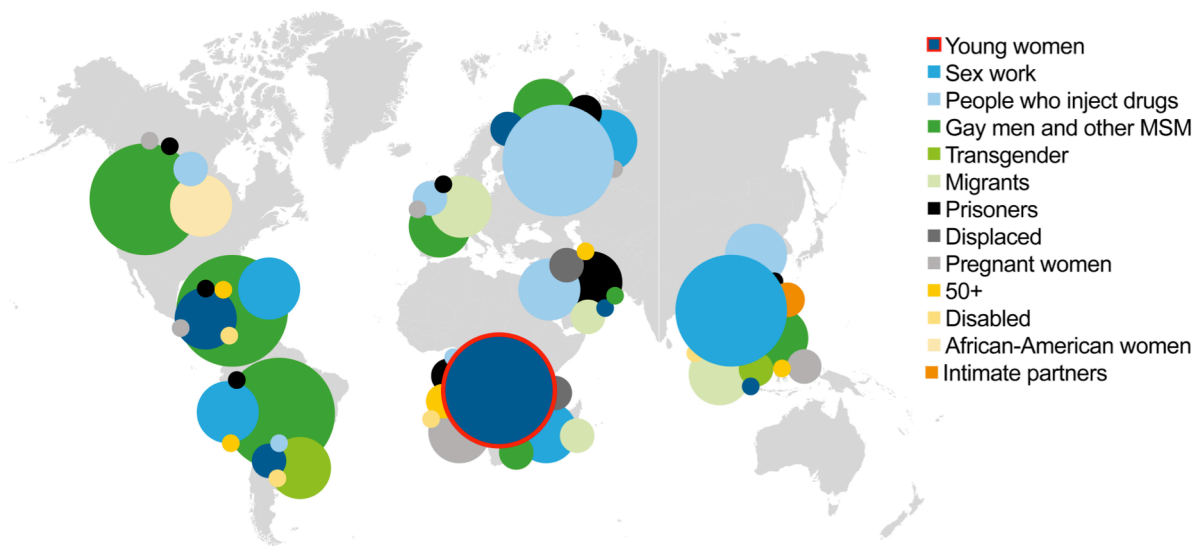


Figure 1.1 Geographic differences in populations living with HIV. Note that the majority of HIV+ young women are in sub-Saharan Africa. Men who have sex with men is abbreviated as MSM. Adapted from the UNAIDS 2014 Gap Report (5).

The number of new infections is decreasing globally, but the incidence rate amongst 18-23 year old women in Durban, South Africa remains around a staggering 7%. The vulnerability of women is exemplified by the rural community of Vulindlela, South Africa where the

prevalence of HIV in girls younger than 14 years is 2% but increases to approximately 50% in 23-24 year old pregnant women (6). It is hard to reconcile this astonishingly high HIV prevalence with estimates of the risk of transmission from an infected man to uninfected women around 0.19% per unprotected sex act (95% CI: 0.0010-0.0037) (7). One interpretation is that particular young women have risk factors that are not captured in the stable serodiscordant couple cohorts that yield such transmission risk estimates. To understand those risk factors, we must improve our understanding of the female genital tract (FGT), the most common site of first exposure to HIV in women.

Brief overview of HIV virology

HIV may very well be the best studied pathogen in the world and a phenomenal amount of detail is known about its structure and life cycle. Briefly, HIV is a retrovirus that comes in two forms: HIV-1 and HIV-2. HIV-2 is mostly found in West Africa and has a lower probability of being transmitted and progressing to AIDS than HIV-1 (8). HIV-1 is the predominant form worldwide and is comprised of three groups, with nearly 98% of circulating viruses belonging to Group M (9); in this thesis HIV-1 will be referred to simply as HIV. Within Group M, Subtype C of HIV-1 is the most common form of HIV in Southern Africa and comprises about 60% of HIV cases worldwide, and Subtype B is the dominant form in Europe and the U.S. (10).

Each HIV virion has a capsid containing: two identical copies of its full-length, 9,749 nucleotide-long RNA genome; reverse transcriptase that lacks 3' exonuclease proofreading activity, leading to about one mutation arising per replication cycle; and integrase (11). Within the viral envelope, which the virion acquires when budding from its host cell, is a protease that is required for the virion to mature and become infective (12). On the surface of the viral envelope are around seven heavily glycosylated trimeric spikes of gp120 with gp41, which determine the target cell tropism by enabling the virus to bind to CD4+ and either the CCR5 or CXCR4 coreceptor. Once HIV fuses to its target cell, the single-stranded RNA genome is

reverse transcribed into cDNA, transported into the nucleus, and integrated into the host genome via the HIV integrase protein. The virus can remain latently integrated in the host genome, or it can actively produce copies of itself by transcribing and translating its viral genes and proteins, which translocate to the cell surface, assemble, and bud off as immature virions (13).

The HIV virion that crosses the genital mucosal barrier to establish a systemic infection in a new host consistently utilizes the CCR5 coreceptor rather than CXCR4 (14). As the focus of this thesis is on HIV transmission and acquisition, the term “target cell” will be used to refer to CCR5+ CD4+ cells.

Anatomy of the female genital tract

The internal female reproductive organs can be divided into upper and lower portions (**Figure 1.2A**). The upper reproductive tract is comprised of the uterus, fallopian tubes, ovaries, and endocervix, of which all but the ovaries are lined by a columnar epithelium that is a single cell thick. The upper tract was traditionally thought to be sterile, but recent evidence calls this assumption into question (15, 16). The lower reproductive tract includes the entire vagina and spans from the vaginal introitus to the cervical os. The muscular vagina is lined by 20-30 cellular layers of stratified squamous epithelium that resembles skin (17, 18). But unlike the skin, the apical layers of the vaginal epithelium, called the stratum corneum, are not tightly adhered and rather form a loose, water-permeable barrier that is regularly sloughed (18). The stratum corneum also acts as a glycogen depot, providing a substrate for both epithelial cells and vaginal *Lactobacillales* bacteria to ferment lactic acid (17). The copious production of lactic acid maintains the vaginal pH at around 4 in women with *Lactobacillus*-dominant microbial communities (19).

The superior end of the vagina is capped by the cervix, which contains a 2-3 cm canal leading to the uterus (20). The cervical canal is lined by goblet cells that produce mucus, a

viscous hydrocolloid that serves as a selectively permeable barrier and lubricant (21). Mucus is also an important component of the innate immune defense against both viruses and bacteria, partially due to its ability to bind the Fc domains of both IgG and IgA immunoglobulins (22). Thus, mucus protects the underlying epithelium, of which there are two types in the cervix: the stratified squamous epithelium of the ectocervix, and the columnar epithelium of the endocervix (**Figure 1.2B,C**). The transition between the ectocervix and endocervix is called the transformation zone, which contains a very high concentration of CD4⁺ T cells, CD8⁺ T cells, and macrophages (23) and is best known as a point of vulnerability in the epithelial barrier that is exploited by human papilloma virus and HIV.

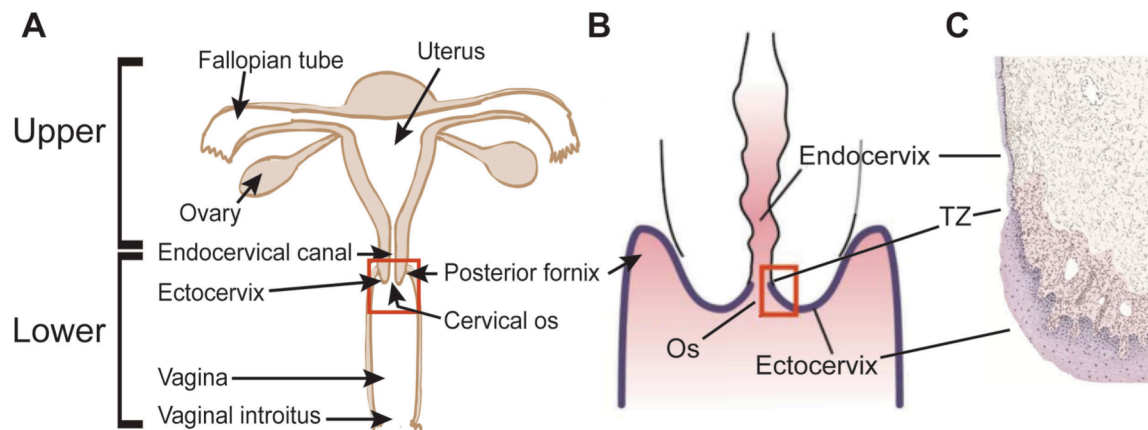


Figure 1.2 Anatomy of the female genital tract. (A) Schematic of the upper and lower female reproductive tract. (B) Magnification of the red box in (A), demonstrating the transition from the ecto to endocervix. (C) Histological magnification of the red box in (B), illustrating the position of the transformation zone (TZ) between the ectocervical stratified squamous epithelium and endocervical single layer of columnar epithelium. (B) and (C) adapted from Coombs *et al. AIDS* 2003 (24).

Physiology of the female genital tract

The physiology of the female reproductive system is controlled by hormones, namely estrogen, progesterone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and gonadotropin-releasing hormone (GnRH). In a woman of reproductive age, GnRH is released by the hypothalamus and acts on the anterior pituitary, which subsequently releases FSH and LH; FSH and LH then stimulate the ovaries to produce estrogen and progesterone (**Figure 1.3**).

These hormones orchestrate the female menstrual cycle, which lasts an average of 28 days and is marked by two major events: the release of a single ovum by the ovaries and the preparation of the uterine endometrium for implantation by the fertilized ovum. If implantation does not occur, the endometrial lining is shed in menstruation. The first 4-5 days of the menstrual cycle are marked by menstruation. The period between the end of menstrual bleeding and ovulation is the follicular or proliferative phase. The last two weeks between ovulation and the initiation of menstrual bleeding is the luteal or secretory phase (**Figure 1.3**).

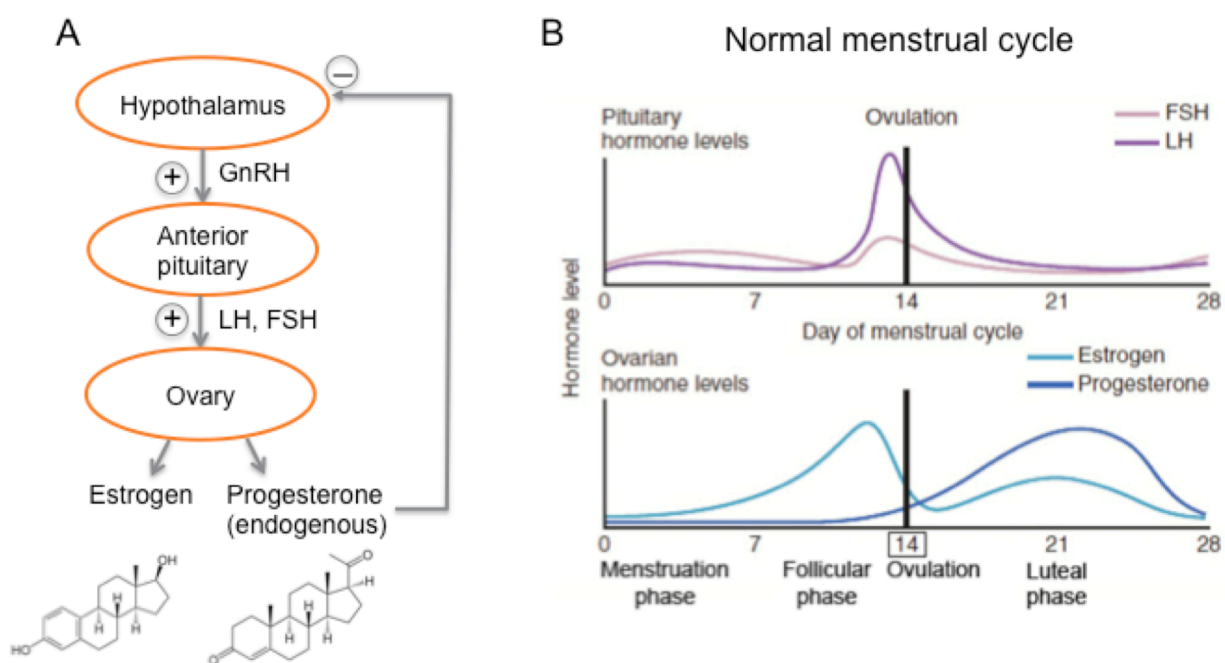


Figure 1.3 Endocrine feedback control of female reproductive levels. (A) The hypothalamus releases gonadotropin releasing hormone (GnRH), which stimulates the anterior pituitary to make luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH then stimulate the ovary to produce estradiol during the follicular phase and progesterone during the luteal phase. (B) Schematic of the female hormone levels throughout the menstrual cycle. Approximately the first four days of the cycle are the menstruation phase, followed by the follicular phase, which lasts up to ovulation. The second half of the cycle, after ovulation to the start of menstruation, is the luteal phase. Adapted from OpenStax College, 2013 (25).

LH and FSH are particularly important for the development and release of the ovum and are at their highest levels prior to ovulation (**Figure 1.3B**), while estrogen and progesterone affect the breasts, bones, adipocytes, and skin in addition to the female reproductive organs. There are three major forms of estrogen in the blood, with the most potent in non-pregnant

females being β -estradiol (26). Estradiol is low (below 100 pg/mL) in the first half of the follicular phase, peaks two days prior to ovulation, and remains at an intermediate concentration (around 150 pg/mL) throughout most of the luteal phase. Alternatively, progesterone is only found at significant levels in the luteal phase (**Figure 1.3B**). Thus, measuring both estradiol and progesterone provide a rough estimate of where a woman is in her menstrual cycle. The pleiotropic effects of estrogen and progesterone on immune responses in the FGT are summarized in **Table 1.1**.

Table 1.1 Effects of estrogen and progestins on aspects of the innate and adaptive immune response. Abbreviations include oral contraceptive pill (OCP), depo medroxyprogesterone (DMPA), cytotoxic T lymphocyte (CTL), and cervicovaginal (CV).

	Estrogen	Progestins
Vaginal epithelial thickness	Estrogen increases epithelial thickness in macaques (27) and humans (28)	Progesterone decreases epithelial thickness in macaques (27) but DMPA does not affect thickness in humans (29)
CCR5 expression on cervical CD4+ T cells	Estradiol treatment of purified peripheral CD4+ T cells decreases HIV cellular entry without altering CCR5 expression levels (30)	<ul style="list-style-type: none"> •OCPs increase CCR5 expression on both cervical CD4+ and CD8+ T cells (31) •Progesterone has been shown to both increase CCR5 expression on blood and cervical T cells (32), and decrease CCR5 expression on blood T cells (33) •DMPA does not alter CCR5 expression on vaginal mucosal CD3 cells (29)
Cytotoxic T cell responses		Inhibition of CTL responses by DMPA (34)
Lymphocyte migration	Decreases T cell migration <i>in vitro</i> (35)	
Antibody production and transport	Estradiol and progesterone decrease IgA, IgG, and secretory component in CV secretions of ovariectomized rats (36)	

Early events in HIV acquisition in the female genital tract

Our understanding of the early events in HIV infection in the FGT are mostly derived from simian immunodeficiency virus (SIV) studies in non-human primate (NHP) models.

Following heterosexual intercourse with an infected partner, mucosal surfaces in the FGT are exposed to an enormously diverse quasispecies of genetically distinct cell-free virions and HIV-infected seminal lymphocytes (37, 38) and yet a poorly understood bottleneck results in a single founder virus establishing the majority of new infections (39-41). SIV infection studies suggest that this single virion undergoes obligate local replication within the mucosal tissue for 1-7 days before disseminating to the draining lymph node and then the blood. Once virus has spread to the periphery, a widespread reservoir of latently infected cells is rapidly established and subsequent viral eradication becomes extremely challenging. This initial period of mucosal replication has been termed a “window of opportunity” for interventions that could contain virus locally and prevent systemic dissemination (42) (**Figure 1.4**).

NHP models indicate that following passage of virus across the cervicovaginal epithelium, the virion encounters susceptible CD4⁺ T cells targets and a small and highly focal founder population of 40-50 infected CD4⁺ T cells is established within 3-4 days of infection. The population of susceptible cells must be large enough to sustain a basic reproductive rate (R_0) >1, such that one infected cell generates more than one newly infected cell, otherwise the infection will burn out (43). Simultaneously, the epithelial cells respond to viral exposure by secreting CCL20 (MIP-3 α), which recruits plasmacytoid dendritic cells that subsequently secrete CCL4 (MIP-1 β), IFN- α , and other chemokines (41). This sets up a delicate balance between interferon-induced protection and chemokine-driven recruitment of susceptible CD4⁺ T cells. Target cell recruitment by innate defenses likely overcomes the low density of target cells at baseline and facilitates cell-to-cell transmission to amplify the initial focus of infected cells (41) (**Figure 1.4**).

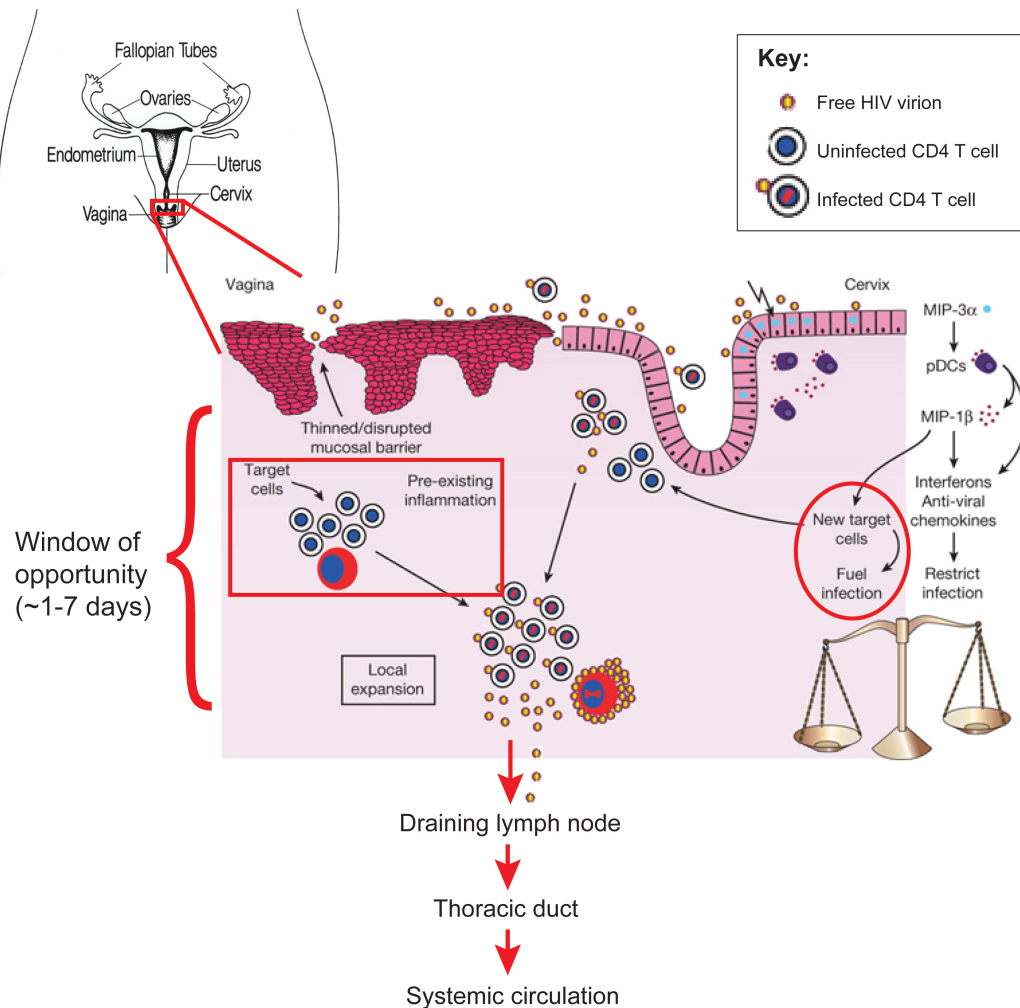


Figure 1.4 Model of the early events in HIV transmission and the important role of genital inflammation. The cervicovaginal epithelium is exposed to both cell-free and cell-associated HIV virions. Only a handful of virions are able to cross the epithelial layer, likely at the cervical transformation zone, endocervix or, if present, breaches in the thick vaginal epithelium. The virus replicates within the genital tissue for several days before being carried via the lymphatics to the draining lymph nodes, then eventually via the thoracic duct to the systemic circulation. Pre-existing genital inflammation increases the number of local CD4⁺ T cell targets and allows faster viral amplification. The innate immune response to viral exposure also recruits additional target cells, further stoking the inflammatory fire. Adapted from A.T. Haase, *Nature*, 2010 (41).

The importance of inflammation in HIV acquisition and progression

Given that effective viral replication and dissemination requires a sufficient density of target cells in the genital mucosa, elevated genital inflammation prior to HIV exposure may facilitate HIV acquisition by increasing the R_0 through the recruitment of activated target cells

and the activation of resident CD4⁺ T cells that support greater HIV replication. In the absence of inflammation, healthy vaginal mucosa mostly contains resting CD4⁺ T cells, which do not support robust HIV replication, unlike activated T cells (44). Activated T cells are commonly identified by the markers CD38, a surface glycoprotein, and HLA-DR, an MHC class II molecule that is not expressed by resting T cells (45). It has been shown *in vivo* that human lymph node HLA-DR⁺ CD38⁺ CD4⁺ T cells have high cell surface concentrations of the HIV co-receptors CCR5 and CXCR4 and contribute to the majority of HIV-1 RNA production (median of 86%) occurring in lymph node CD4⁺ T cells (46). Specifically in the genital tract, the intracellular concentration of SIV RNA in activated (HLA-DR⁺) and proliferating (Ki67⁺) endocervical T cells was 4-7 times higher than in resting T cells (47). Thus, the frequency and activation state of CD4⁺ T cells in the FGT may have a critical role in determining the susceptibility to HIV acquisition.

There are also several *in vivo* studies that support the importance of inflammation in HIV acquisition risk. In an SIV vaccine study with intrarectal challenges, macaques who became infected had twice as many CCR5⁺ HLA-DR⁺ CD4⁺ T cells in the rectum compared to those who remained uninfected, and there was a significant correlation between the pre-infection frequency of activated CCR5⁺ CD4⁺ T cells in the rectal mucosa and the day 7 plasma viral load (48). In serodiscordant couples, HIV acquisition was associated with elevated serum CXCL10 and IL-10 concentrations (49). In South African women enrolled in the CAPRISA 004 study, plasma TNF- α and IL-12p70 and cervicovaginal IL-1 α , IL-1 β , TNF- α , CCL20 (MIP-1 α), CCL4 (MIP-1 β), and IL-8 were higher in women who acquired HIV (50, 51). The converse is also true; low systemic immune activation seems to have a protective effect in cohorts of highly exposed but seronegative hemophiliacs (52) and individuals in discordant couples (53).

Inflammation continues to play an important role even after HIV transmission has occurred. In the genital tract, increased proinflammatory cytokine levels in cervicovaginal lavages (CVL) collected in early infection are also associated with a worse disease course, as

measured by higher viral load set points and CD4+ T cell depletion (54). In the blood, activation levels of CD4+ and CD8+ T cells were strongly correlated with decreased patient survival (55). In the gut, which harbors the vast majority of activated T cells in the body, CCR5+ CD4+ T cells are preferentially depleted within the first few weeks of infection (56). In fact, some studies suggest that the reduction in the number of Th17 cells in the gut leads to a decrease in the local IL-22 concentration, which causes the epithelial barrier to weaken. This leaky epithelium is thought to enable microbial translocation, thereby causing further systemic immune activation and increased target cell availability via TLR4 stimulation by translocated gut bacteria (57). Thus, both cellular and soluble inflammation affect HIV acquisition and progression.

Social and behavioral risk factors for HIV acquisition in young women

The risk of HIV transmission from an infected man to an uninfected woman is determined by the seminal viral load of the infected male, condom use, and the susceptibility of the female partner (7).

The conventional “ABCs” of HIV prevention are “abstinence, be faithful, and use condoms”. Abstinence education is controversial and challenging from a policy perspective, but delaying sexual onset to an older age undoubtedly decreases a woman’s risk of HIV acquisition (58). Older women are more educated, financially independent, and assertive in negotiations with their sexual partners (59).

Regarding “being faithful”, it is not unusual for men in sub-Saharan Africa to have multiple sexual partners; for example, up to 55% of men in Lesotho report having more than one regular partner or spouse (60). Multiple partners expand sexual networks and obviously increase the risk of HIV acquisition for everyone in the network. The tolerance of multiple partners is thought to come from cultural norms, the institutionalized authority of men over women in marriage, and more subtle control afforded to men from earning a higher income (61). Some women rely on their partners for financial support, creating a power dynamic that further

complicates negotiations for safe sex (62). It is also common in sub-Saharan Africa for younger women to have relationships with older men, who are more likely to have multiple partners and have HIV (59). This practice is thought to be the basis of the left shift in HIV prevalence curves for young women compared to young men (Figure 1.5) (1).

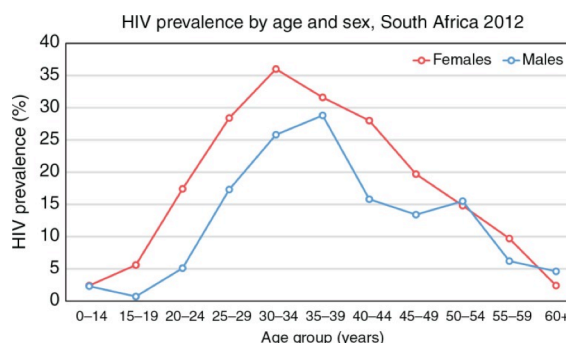


Figure 1.5 Differences in HIV prevalence amongst young men and women. Adapted from Dellar *et al.*, *JIAIDS*, 2015 (1).

The third component of the ABCs is using a condom. Male condoms are widely available and often free in clinics in sub-Saharan Africa, but adherence is controlled by the man. While female condoms are very effective, they are rarely used because they are harder to find and people are unfamiliar with how to use them (63). Stigma remains a general barrier to condom use, as demanding to use a condom may be perceived as a subtle confession of having HIV or another sexually transmitted infection (59).

Biological risk factors for HIV acquisition in young women

There are numerous biological factors that are associated with an increased HIV acquisition risk in women. The most common biological risk factors are sexually transmitted infections, bacterial vaginosis, and injectable progestin-only hormonal contraceptives.

Sexually transmitted infections

The four sexually transmitted infections most robustly linked to HIV acquisition are herpes simplex virus 2 (HSV-2), gonorrhea, chlamydia, and *Trichomonas vaginalis* (64).

Herpes simplex virus type 2

Herpes simplex virus is a member of the herpes virus family and comes in two forms, called type 1 and type 2. HSV-1 primarily causes an oropharyngeal infection, while HSV-2

infects the vulva and penis, although both types of HSV can infect both oral and genital sites. Both establish lifelong infection in the host, characterized by periods of latency in the sensory ganglion cells and recurrence in the skin innervated by the infected neurons (65). While the skin lesions can be very painful, most people who are seropositive for HSV-2 are asymptomatic (66).

An active HSV-2 ulcer generates breaches in the epithelial barrier that are thought to facilitate HIV entry into the genital dermis and increase the risk of HIV infection by 2 to 4-fold (67). After HSV-2 lesions have healed, there remains an inflammatory focus that includes CCR5+ CD4+ T cells, which may contribute to increased acquisition rates even in the absence of lesions (68). Thus, both active and healed HSV-2 lesions impact HIV risk.

Chlamydia trachomatis

Chlamydia trachomatis is a gram-negative obligate intracellular bacterium that has a unique biphasic replicative cycle, existing as a tough, infectious elementary body (EB) when extracellular survival is required, and a fragile, non-infectious reticulate body (RB) when inhabiting an intracellular niche (69). The EB is able to enter the host epithelial cell, differentiate into a RB, replicate to form 100-1000 more RBs, and recondense those RBs back into EBs (69). About 48 hours after entering their host cell, newly produced elementary bodies can be released by either lysing their host or through exocytosis (69, 70).

C. trachomatis poses a significant risk to women if untreated, due to its ability to cause pelvic inflammatory disease (PID) and eventually infertility by scarring the fallopian tubes (71). Chlamydia is easily treated with a short course of antibiotics, but women infrequently seek treatment due to the asymptomatic nature of most infections. However, clinical signs of local tissue damage and an inflammatory response are often present on exam, including a mucopurulent discharge from the cervical os and a friable cervical epithelium that bleeds easily (71).

Women with active *C. trachomatis* infections have elevated levels of numerous cytokines including IL-1 β , IL-6, TNF- α , CXCL10 (IP-10), CCL20 (MIP-1 α), CCL4 (MIP-1 β), and RANTES in cervicovaginal lavages compared to women without any STIs or bacterial vaginosis (BV) after adjusting for coinfections and injectable hormonal contraceptive use (72). While CD4+ and CD8+ T cells are activated in response to chlamydia, both responses are short-lived and do not provide durable immunological memory, possibly because of direct bacterial inhibition (73). Due to this ineffective immune response, repeated untreated chlamydia infections result in chronic progressive inflammation, fibrosis, and scarring (69).

Neisseria gonorrhoeae

Like chlamydia, *Neisseria gonorrhoeae* is a gram-negative bacterium, over 50% of infected women are asymptomatic, and primary infection provides little protective immunity against repeated infection. Unlike *C. trachomatis*, *N. gonorrhoeae* is not an obligate intracellular bacterium, rather it uses its numerous pili and Opa proteins to adhere to nonciliated epithelial cells (74). After attachment, it triggers the host cell to phagocytose itself, and then manages to exit the cell through the basal surface (74). Within the mucosa, *N. gonorrhoeae* utilizes an arsenal of evasion mechanisms to inhibit complement activation, resist intracellular killing by phagocytes, and escape antibody recognition by antigenic variation (75). Given these sophisticated evasion mechanisms, perhaps it is no surprise that women with uncomplicated gonococcal infections do not have detectable increases in local pro-inflammatory cytokine levels or anti-gonococcal antibodies (72, 76, 77).

Trichomonas vaginalis

T. vaginalis (TV) is a protozoan flagellate that infects the vulva, vagina, and cervix in women and the prostate, seminal vesicles, and urethra in men. Infections are often asymptomatic but can cause a thin, white discharge accompanied by genital itching and burning (78). As a parasite, TV sometimes carries a virus called TVV (*Trichomonas vaginalis* virus) (79),

which amplifies the inflammatory reaction to TV (80). Without stratification based on TVV presence, TV is not significantly associated with any CVL cytokine changes, though there is a trend towards higher concentrations of IL-8 with TV (72, 81).

Bacterial vaginosis

Even in the absence of a frank STI, altered vaginal microbiota in the context of bacterial vaginosis (BV) increases the risk of HIV acquisition by approximately 60% (82). Definitions for BV vary, but the clinically utilized Nugent score assesses the loss of the dominant gram-positive *Lactobacillus* species and presence of small and curved gram-variable rods that are often *Gardnerella vaginalis*, *Mobiluncus*, and *Prevotella* (**Figure 1.6**) (83). Over half of all women with BV are asymptomatic, but it is the most common cause for vaginal discharge. Using the Ansel clinical criteria of a vaginal pH above 4.5, increased watery vaginal discharge, presence of clue cells, and positive amine or whiff test, the estimated prevalence of BV in women of reproductive age in the United States is 29.2%, with significant variation among racial groups (with non-Hispanic black women having the greatest prevalence of 51.4%) (84). Estimates for the prevalence in South Africa range from 15 to 70% (82, 85).

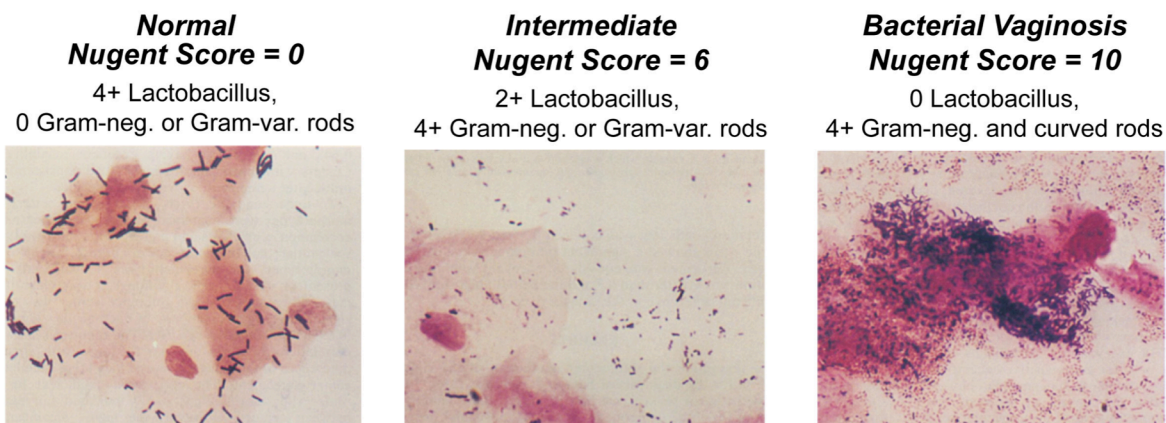


Figure 1.6 Examples of Gram stained vaginal smears with Nugent scores. Adapted from Nugent et al. *Journal of Clinical Microbiology* (83).

Hormonal contraceptives

Injectable progestin-only contraceptives (IPCs) are a particularly popular contraceptive form in sub-Saharan Africa. IPCs have a minimal failure rate because they are extremely convenient to use, requiring a single injection once every two (for norethisterone enanthate, marketed as Nur-isterate) or three months (for depot-medroxyprogesterone/ DMPA, marketed as Depo-Provera). IPCs perform their contraceptive function by inhibiting the LH surge that triggers ovulation (**Figure 1.7**), increasing the thickness of cervical mucus to block the passage of sperm through the endocervical canal, and changing the endometrial lining to impair implantation (86, 87). They subsequently suppress the endogenous production of estradiol and progesterone (**Figure 1.7**).

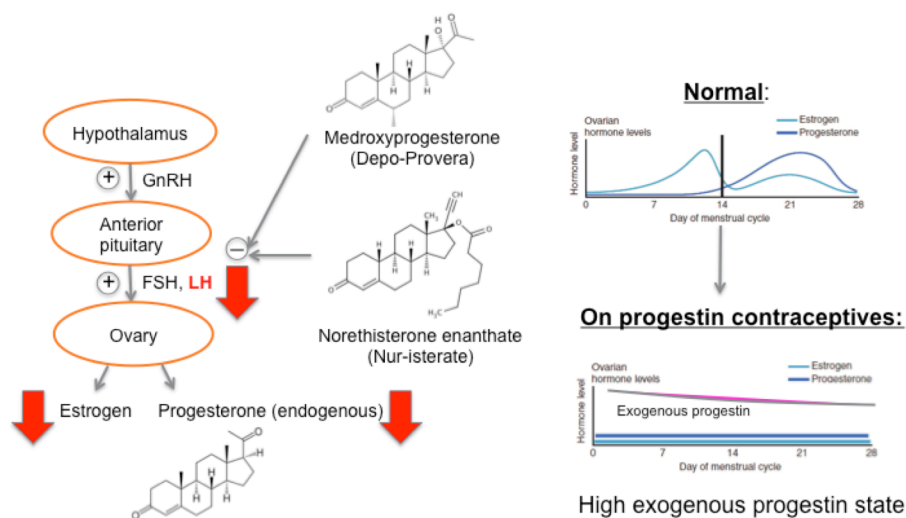


Figure 1.7 Suppressive effects of IPCs on endogenous estrogen, progesterone, and luteinizing hormone (LH) levels.

Hormonal contraceptives are a controversial risk factor for HIV acquisition. Many prospective studies in sub-Saharan Africa have sought to determine whether DMPA is associated with an increased HIV acquisition risk. The results are nearly evenly split (**Table 1.2**), though a recent meta-analysis found a pooled hazard ratio of 1.4 and 95% CI of 1.16-1.69 for DMPA use (88). There is less data available for Nur-isterate and oral contraceptive pills but the studies to date do not demonstrate an increased HIV risk compared to not using a family

planning method (88). Several of these studies follow high-risk cohorts of sex workers (89, 90) and serodiscordant couples in stable relationships (91), but their unique sexual behaviors and underlying risk factors may hinder the general applicability of the study conclusions. Inclusion criteria also differ amongst studies, with the inclusion of older women between the ages of 35 to 49 in several studies (92-94). Older women are more likely to use DMPA and be in a stable partnership, thus the effect size of DMPA might seem diminished due to less risky behavior. These confounders, which are inherent to observational studies, have led to the preparation of a highly controversial randomized control trial called the Evidence for Contraceptive Options and HIV Outcomes (ECHO) trial, which will randomize 7,800 women to use Depo-Provera, a hormonal implant called Janelle, or a copper intrauterine device. Critics of the proposed \$48 million trial do not believe that the study is ethical because it does not meet the criteria of clinical equipoise, meaning that there is genuine uncertainty about which treatment will be the most risky. Advocates consider the trial to be the only way to definitively answer the question of whether DMPA increases HIV risk.

Table 1.2 Summary of prospective studies that tested the hypothesis that DMPA use was associated with an increased HIV acquisition risk. The hazard ratio (HR) and 95% confidence interval are shown for each study finding an increased risk.

Demonstrated an increased HIV acquisition risk with DMPA use	Found no difference in risk with DMPA use
Crook <i>et al.</i> 2013. HR 1.49 [1.06 - 2.08] (95)	McCoy <i>et al.</i> 2013. (93)
Heffron <i>et al.</i> 2012. HR 1.98 [1.06 - 3.68] (91)	Morrison <i>et al.</i> 2012. (96)
Wand <i>et al.</i> 2012. HR 1.72 [1.19 - 2.49] (97)	Reid <i>et al.</i> 2010. (94)
Baeten <i>et al.</i> 2007. HR 1.73 [1.28 - 2.34] (89)	Myer <i>et al.</i> 2007. (92)
Lavreys <i>et al.</i> 2004 ^a . HR 1.8 [1.4 - 2.4] (90)	Kiddugavu <i>et al.</i> 2003. (99)
Martin <i>et al.</i> 1998 ^a . HR 2.0 [1.3 - 3.1] (98)	Kapiga <i>et al.</i> 1998. (100)
	Bulterys <i>et al.</i> 1994. (101)

^aUtilized the same cohort. Lavreys extended the analysis to include the period of 1997-2003.

Unlike the clinical studies in humans, the non-human primate (NHP) model unequivocally demonstrates an increased risk of HIV acquisition with DMPA use (102-104). In fact, DMPA is routinely used to increase infection efficiency, synchronize hormone levels, halt menstruation, and thin the epithelial lining of vagina prior to intravaginal SIV and HIV infections

in NHP and humanized mouse models, respectively. The ability to extrapolate these findings to humans is hindered by differences in viral titer (very high titers are used in NHP challenge models to achieve high per-inoculation infection rates), the form of the virus (NHP are challenged with free virions in culture media, while women are infected by both cell-associated and cell-free virus in semen), and differences in the doses and physiologic responses to DMPA treatment (105).

The final complication is that medroxyprogesterone acetate (MPA) and norethisterone (NET), which is the metabolically-active hydrolyzed form of norethisterone enanthate, do not merely replace the biological effects of endogenous progesterone due to their differential effects on steroid hormone receptors (**Table 1.3**) (106). Progesterone, MPA, and NET all bind to the progesterone receptor but both IPCs have higher binding affinities than endogenous progesterone. Unlike progesterone, MPA and NET both bind to the androgen receptor, albeit very weakly (**Table 1.3**). Finally, the glucocorticoid receptor activity of MPA may be especially important due to the pleiotropic effects of glucocorticoids on nearly every organ system and lymphocytes (107, 108).

Table 1.3. Relative binding affinities of progesterone, MPA, and NET to steroid hormone receptors. (+) indicates the magnitude of the effect, (±) indicates a weak effect, and (-) indicates a negligible effect. Adapted from (106).

Progestin:	Progesterone Receptor	Estrogen Receptor	Androgen Receptor	Glucocorticoid Receptor
Progesterone	+	–	–	+
MPA	+++	–	±	+++
NET	++	–	+	–

In summary, the risk factors for HIV acquisition are a complex set of social, behavioral, and biological factors. The underlying mechanisms by which the numerous biological factors increase HIV susceptibility are uncertain, and this thesis focused on developing a better understanding of the inflammatory properties of cervicovaginal bacteria and injectable progestin-only contraceptives.

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Chapter 2: Characterizing cervicovaginal bacterial communities and their strong association with genital pro-inflammatory cytokines in HIV-uninfected and HIV-infected South African women

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Attributions

M.N.A. designed the mucosal collection protocols and the experiments with the help of D.S.K., performed the CVL cytokine measurement, data analyses, nucleic acid extraction and bacterial sequencing. B.A.B. and E.H.B assisted with the nucleic acid extraction and bacterial sequencing; C.G. performed the viral load measurements; M.N.A., K.E.D., N.P. and D.S.K. performed FACS analysis; K.E.D. and A.M. collected behavioral data; M.S.G. provided advice for statistical analyses; C.H., H.H.V., and B.D.W. provided advice with experimental design and data analysis.

Introduction

The female genital tract (FGT) maintains a finely-tuned immune response that balances reproductive tolerance with protection against genital infections. While inflammatory responses are beneficial and necessary for effectively eliminating several sexually transmitted infections (STIs), the presence of elevated genital inflammation in women prior to HIV exposure paradoxically increases the risk of disease acquisition (1, 2). Studies of the microbial causes of this inflammation have primarily focused on established pathogens such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HSV-2, and *Trichomonas vaginalis*. The potential role of commensal cervicovaginal bacteria, which number $\sim 10^8$ per gram of vaginal fluid (3), in modulating immune responses in the FGT is largely unknown.

The bacterial microbiome of the healthy FGT has traditionally been thought to be exceedingly simple, predominated by a single *Lactobacillus* species in the majority of white premenopausal women (4-8). These species of *Lactobacillus* benefit the host by inhibiting the growth of pathogenic bacteria and fungi through the production of bacteriocins, lactic acid, and hydrogen peroxide (9, 10). Bacterial vaginosis (BV) is a symptomatic alteration of microbial communities in this ecological niche in which species of *Lactobacillus* are replaced by *Gardnerella* and *Mobiluncus* species. BV is associated with a 1.5–2-fold increased risk of

acquiring *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and HIV (11, 12), which raises the question of whether specific genital microbial communities in asymptomatic women may more broadly modulate disease susceptibility.

In addition to modulating disease acquisition risk, microbial changes have also been implicated in accelerating the progression of HIV to AIDS (13-15). Disease acceleration is thought to occur through two main pathways. The first is severe compromise of the gut mucosal epithelial barrier by HIV-mediated killing of protective CD4⁺ T cells, leading to impairment of barrier function, microbial translocation and chronic systemic inflammation (16-18). The second pathway is decreased microbial stability and shifts in the gut microbial composition towards more pro-inflammatory, pathogenic communities that add fuel to the inflammatory fire (13, 19-21). The mechanisms leading to this gut microbial change in HIV infection are unknown, but the loss of effector CD4⁺ T cells leading to a failure to mount immune responses to pathogenic bacteria is one obvious possibility (22). Even less is known about changes in the vaginal microbiome in HIV infection, though studies published to date either failed to see a difference (23, 24) or saw less *Lactobacillus crispatus* in HIV-infected women (25). Longitudinal, high-frequency bacterial characterization throughout the acute and chronic phase of HIV infection could provide more definitive evidence for HIV-induced microbial changes and provide important insight into the impact of host immune responses on microbial composition.

To determine the contribution of cervicovaginal microbiota to genital inflammation, we evaluated a cohort of young South African women. Only a minority of HIV-negative study participants had *Lactobacillus*-dominant cervicovaginal communities, despite a lack of clinical symptoms, redefining what is considered to be normal in this region. We found a strong cross-sectional and longitudinal *in vivo* relationship between high-diversity bacterial communities lacking *Lactobacillus* dominance and genital pro-inflammatory cytokine levels. We then compared the microbial community members of women who remained HIV-negative and those

who became infected. Finally, we determined the resilience of the cervicovaginal microbiome during the dramatic perturbation of acute HIV infection.

Results

A minority of South African women in FRESH had *Lactobacillus* dominant genital communities

Participants were recruited through the Females Rising through Education, Support, and Health (FRESH) program, a prospective, 12-month observational study in Durban, South Africa. Participants had to be female, HIV-negative, nonpregnant, sexually active, and between the ages of 18 and 23 to be enrolled. Study volunteers attended classes twice a week that focused on empowerment, job skills training, and health education. Participants had a finger prick blood draw for HIV RNA testing at each visit, and completed a behavioral risk questionnaire and received a pelvic exam and venous blood draw every three months. Despite intensive counseling and STI testing, the HIV incidence in the cohort was 7%. If a participant acquired HIV during the study, she received further counseling, more frequent pelvic exams and blood draws, and was offered anti-retroviral therapy, as explained in more detail in the Methods section.

We began by assessing the baseline bacterial microbiome in participants from the FRESH study. Following isolation of nucleic acid from cervical swabs, we sequenced variable region 4 (V4) of the bacterial 16S rRNA gene to assess bacterial abundances (26) (**Figure 2.1**). We chose to sequence the V4 region of the 16S rRNA gene due to the availability of extensively validated barcoded primers that are optimized for Illumina sequencing. While the V4 region has tremendous discriminatory power between most genera, it does not distinguish between all *Lactobacillus* species at a 97% operational taxonomic unit (OTU) level due to sequence similarity in that region.

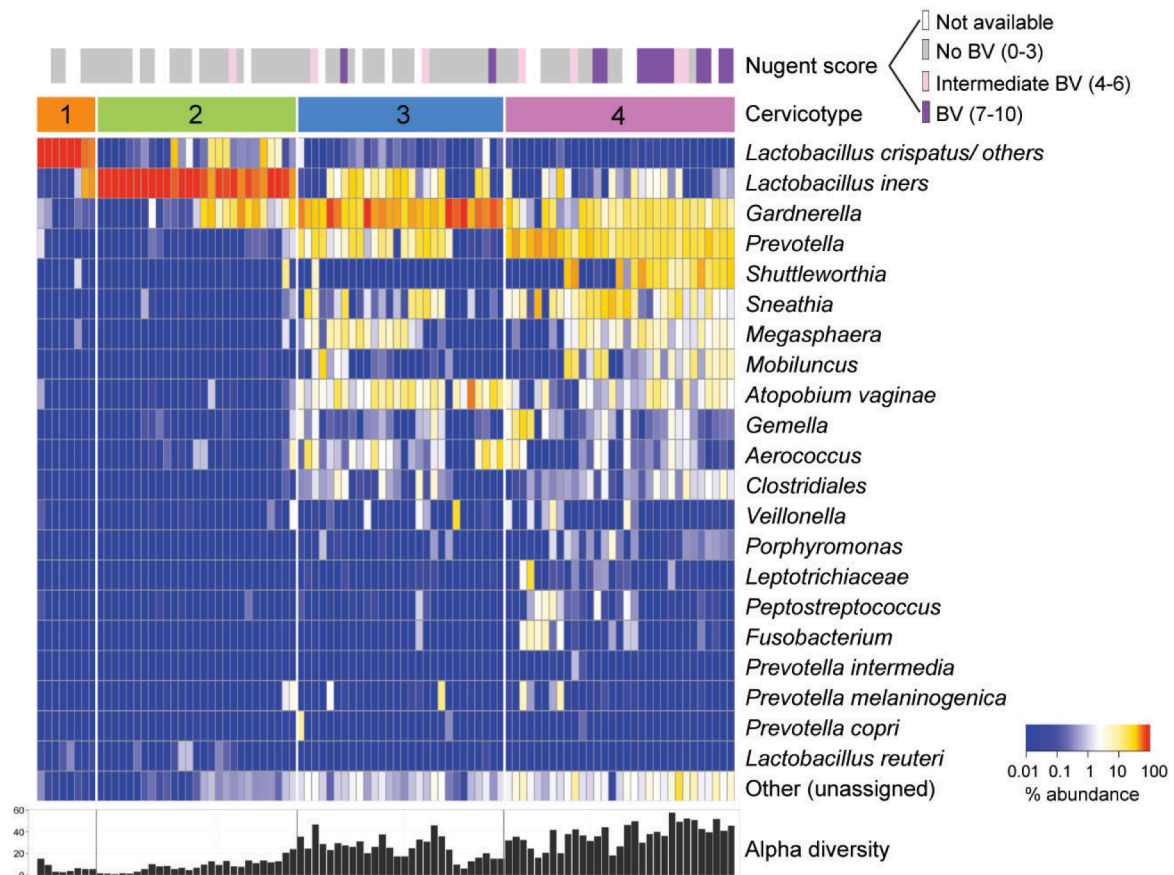


Figure 2.1 16S analysis of cervical swabs reveals low *Lactobacillus* abundance and four distinct bacterial community structures. Heatmap of bacterial taxa identified by 16S V4 sequencing of cervical swabs collected from 94 women. Cervicotypes (CTs) were determined based on the dominant species: *Lactobacillus crispatus* (CT1), *L. iners* (CT2), *Gardnerella* (CT3), and mixed microbial communities containing *Prevotella* (CT4). Nugent scores and alpha diversity are also shown.

We clustered the observed microbial communities into four distinct community types based on the dominant bacterial species, herein referred to as “cervicotypes” (CTs). While there has been some backlash against defining community types in the gut, our cervicotypes are primarily taxon-based classifiers as opposed to the cluster-based classification of “enterotypes” (27), which renders cervicotypes more robust and biologically meaningful. CT1 was primarily composed of non-

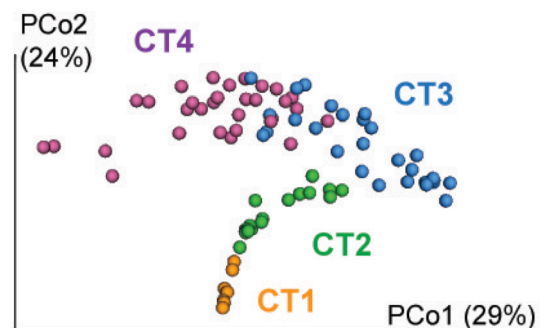


Figure 2.2 Principal coordinates analysis using the weighted UniFrac distance metric on the same 94 samples, colored by cervicotype (CT).

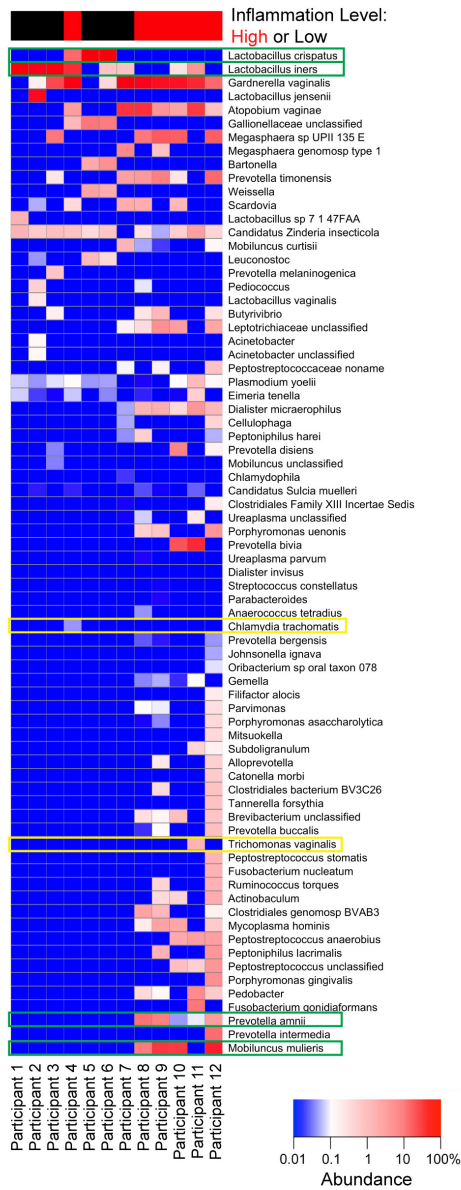
iners Lactobacillus (i.e. higher percentage of sequencing reads from non-*iners Lactobacillus* than *L. iners*, *Gardnerella*, or *Prevotella*); CT2 was *Lactobacillus iners* dominant; CT3 had *Gardnerella* dominance; and CT4 lacked a consistent dominant species but communities all included *Prevotella* (**Figure 2.1**). Visualization of the same samples using a principal coordinates plot, which represents the phylogenetic distance between samples, supported the dominance-based clustering, though CT3 and CT4 are clearly a continuum (**Figure 2.2**).

We found that only 37% of participants had *Lactobacillus* dominant cervicovaginal communities. This is in contrast to published reports of white and black women in developed countries in which 90% and 62% of women respectively demonstrated *Lactobacillus* dominance (4, 5). Of those women with *Lactobacillus* dominance in our study, 77% primarily had *Lactobacillus iners* (CT2). *L. iners* is biologically distinct from other *Lactobacilli* due to its unique adaptation to survive with diverse community members (28) and greater pathogenic potential (29, 30).

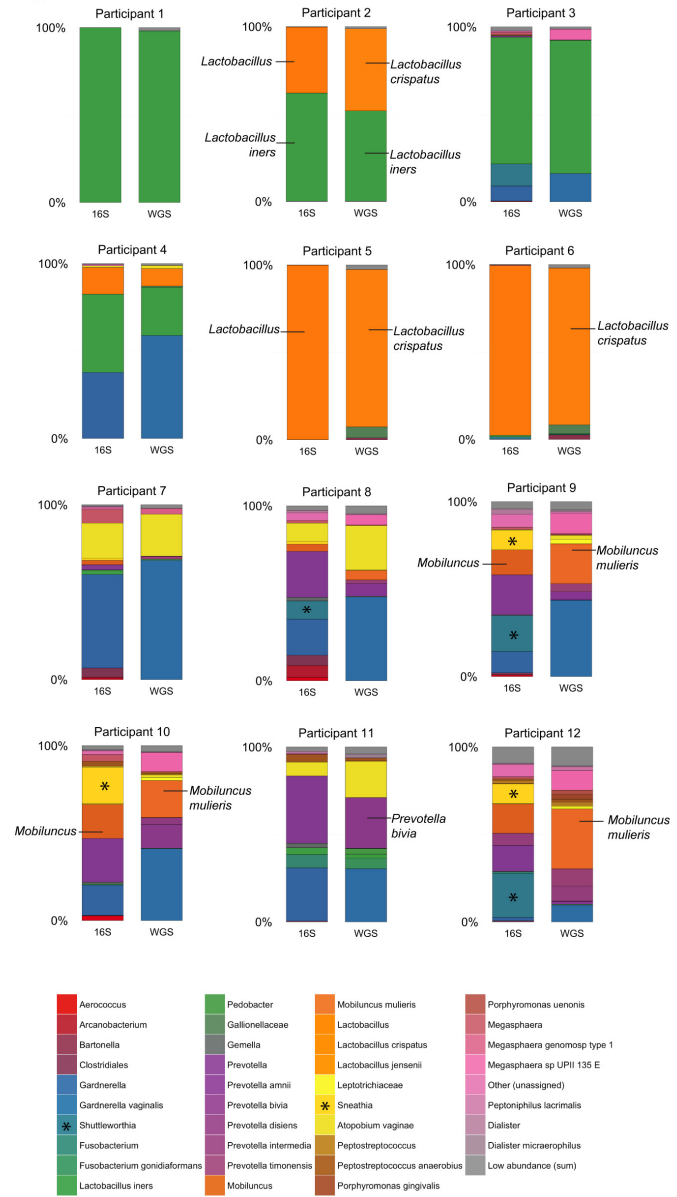
Of the 63% of women in our cohort who did not have *Lactobacillus* dominance, 45% had *Gardnerella* dominant communities (CT3). The remaining 55% of women did not have a consistent predominant bacterial taxon, though each community was found to have at least 10% *Prevotella* abundance (CT4). Additionally, only half of the women in CT4 had BV, as measured by the Nugent criteria, which assesses a Gram-stained vaginal wet prep based on bacterial morphology; none of these women reported symptoms. Overall, the bacterial community structures seen in these asymptomatic young South African women are characterized by a predominance of communities with high ecological diversity and low abundance of non-*iners Lactobacillus*.

Figure 2.3 Summary of metagenomic taxonomic identification using whole-genome shotgun sequencing and oligotyping. (A) Heatmap of metagenomic taxonomic identification using the MetaPhlAn2 tool, with median clustering (dendrogram not shown). Six women with the highest pro-inflammatory cytokine levels (red) and six with the lowest levels (black) were chosen for metagenomic profiling. Taxa with abundances greater than 0.01% in any sample were shown. *Chlamydia trachomatis* and *Trichomonas vaginalis* were only detected in women who were PCR positive (yellow box). Species tested *in vitro* are highlighted in green. (B) Comparison of 16S (left) and whole-genome shotgun sequencing (WGS; right) taxonomic identification for all twelve participants for whom both methods were used. Note that MetaPhlAn2, the program that we used for bacterial classification of the WGS reads, does not include *Sneathia* or *Shuttleworthia* in its reference database (due to the absence of full genome sequences for these bacteria), and thus cannot assign those genera. *Sneathia* or *Shuttleworthia* reads from 16S sequencing are marked with an asterisk. (C) Oligotyping was performed on the reads that QIIME assigned to *Lactobacillus* (not *inerts*) from women in CT1.

A



B



C

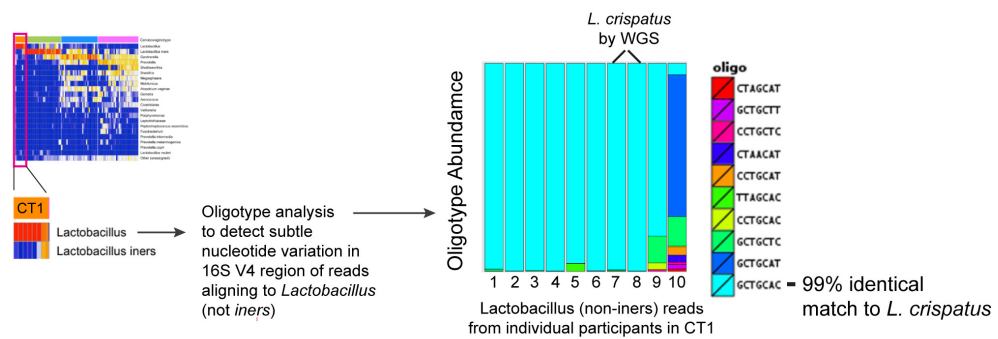


Figure 2.3 (Continued)

We next confirmed the 16S sequencing results and resolved specific community members at a species level by performing whole-genome shotgun (WGS) sequencing on cervical bacterial DNA from a subset of 12 women representing all four CTs (**Figure 2.3A**). Six women were among those with the highest pro-inflammatory levels, and six were among the lowest. WGS has proven useful for species-level taxonomic resolution when different bacterial species share nearly identical 16S rRNA variable regions (31, 32). The taxonomic classification by WGS had good agreement with 16S-based classification and STI PCR results (**Figure 2.4 and 2.3B**). The additional species-level resolution provided by WGS also revealed that both of the women with CT1 analyzed by WGS had *Lactobacillus crispatus* dominant microbial communities (**Figure 2.4A and 2.3C**). We extended the analysis to every woman with CT1 using taxonomic identification by oligotyping, a method which detects patterns in subtle nucleotide variations within the 16S gene (33), and found that nearly all women had identical sequences that aligned to *L. crispatus*, further validating that CT1 is mostly comprised of *L. crispatus*.

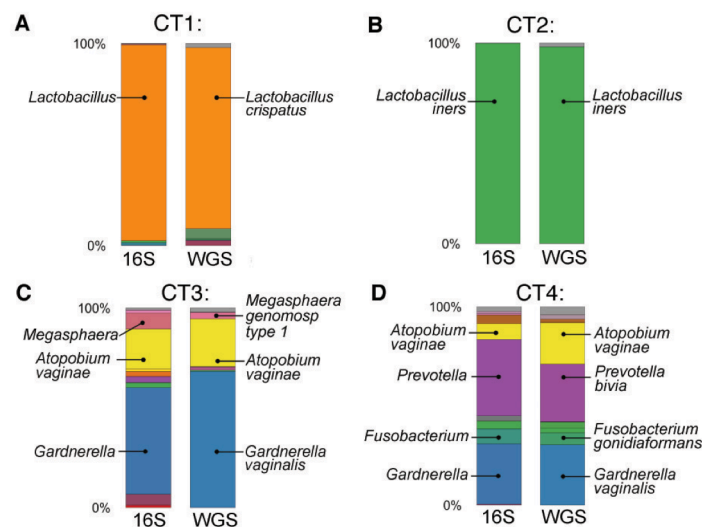


Figure 2.4 Comparison of 16S (left) and whole-genome shotgun sequencing (WGS; right) taxonomic identification for all CTs. Participant #6 represents CT1 (A), #1 represents CT2 (B), #7 represents CT3 (C), #11 represents CT4 (D).

Table 2.1 Contraceptive usage, active sexually transmitted infections, condom use, and sexual behavior are not associated with the bacterial community state.

	All n=94	CT1 n=8	CT2 n=30	CT3 n=25	CT4 n=31	
STIs:	n (%)	n (%)	n (%)	n (%)	n (%)	
Gonorrhea	1 (1%)	1 (12%)	0 (0%)	0 (0%)	0 (0%)	p=0.08391 ^a
Chlamydia	9 (9.6%)	0 (0%)	4 (13%)	2 (8%)	3 (9.7%)	p=0.8855 ^a
Trichomonas	6 (6.4%)	0 (0%)	1 (3%)	3 (12%)	2 (6.5%)	p=0.5796 ^a
HSV2	3 (3.1%)	0 (0%)	0 (0%)	1 (4%)	2 (6.5%)	p=0.6197 ^a
HSV1	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	p=1.000 ^a
No STIs	71 (75%)	7 (88%)	25 (83%)	19 (76%)	20 (64%)	p=0.3609 ^b
History of STIs (self-reported):						
Yes	18 (19%)	1 (12%)	10 (33%)	4 (16%)	3 (9.7%)	p=0.09167 ^c
No	72 (77%)	7 (88%)	18 (60%)	19 (76%)	28 (90%)	
Not available	4 (4.2%)	0 (0%)	2 (6%)	2 (8%)	0 (0%)	
Experiencing STI symptoms at time of exam: ^d						
Yes	2 (2.1%)	0 (0%)	1 (3.3%)	0 (0%)	1 (3.2%)	p=1 ^c
No	89 (95%)	8 (100%)	28 (93.3%)	24 (96%)	29 (93.5%)	
Not available	3 (3.1%)	0 (0%)	1 (3.3%)	1 (4%)	1 (3.2%)	
Contraceptive:						
No family planning	43 (46%)	4 (50%)	14 (47%)	10 (40%)	15 (48%)	p=0.9474 ^c
DMPA/ Nuristerate	42 (45%)	4 (50%)	14 (47%)	12 (48%)	12 (38%)	
OCP/ Non-hormonal contra.	9 (9.6%)	0 (0%)	2 (6%)	3 (12%)	4 (13%)	
Condoms in 30 days:						
Always	16 (17%)	3 (37%)	3 (10%)	4 (16%)	6 (19%)	p=0.2775 ^c
Sometimes	29 (31%)	4 (50%)	10 (33%)	7 (28%)	8 (25%)	
Never	27 (29%)	0 (0%)	12 (40%)	6 (24%)	9 (29%)	
No response/ no sex in past 30 days	20 (21%)	1 (13%)	3 (10%)	8 (32%)	8 (26%)	
# of sex episodes in past 7 days:						
None	47 (50%)	4 (50%)	10 (33%)	15 (60%)	18 (58%)	p=0.2633 ^c
One or more	43 (46%)	4 (50%)	17 (57%)	9 (36%)	13 (42%)	
Not available	4 (4%)	0 (0%)	3 (10%)	1 (4%)	0 (0%)	
# of regular sex partners in last 30 days:						
None	39 (41%)	3 (38%)	7 (23%)	15 (62%)	14 (45%)	p=0.05377 ^c
One	51 (54%)	4 (50%)	21 (70%)	9 (38%)	17 (55%)	
More than one	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Not available	4 (5%)	1 (12%)	2 (6.6%)	1 (4%)	0 (0%)	
# of casual sex partners in last 30 days:						
None	88 (94%)	7 (88%)	26 (93%)	24 (96%)	31 (100%)	p=0.3131 ^c
One	2 (21%)	0 (0%)	2 (6.6%)	0 (0%)	0 (0%)	
More than one	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	
Not available	4 (5%)	1 (12%)	2 (6.6%)	1 (4%)	0 (0%)	
Days since last sexual act (Median, IQR):	8 [3, 26]	8 [6, 21]	6 [2, 19.75]	16.5 [4, 37]	14 [5, 52]	p=0.275 ^e
# of sex episodes in past 30 day (Median, IQR):	2 [0, 4]	2 [1, 4]	2 [1, 4]	1.5 [0, 3]	1 [0, 4]	p=0.289 ^e
# of anal sex acts in past 30 days (Median, IQR):	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	0 [0, 0]	p=0.621 ^e
Age of participant (Median, IQR)	21 [20, 22]	21 [19.25, 22]	21 [20.75, 23]	21 [20, 22]	21 [20, 23]	p=0.5631 ^e

a. Fisher's exact test, comparing each STI to those without that STI.

b. Fisher's exact test, comparing those with any STI to those without an STI.

c. Fisher's exact test, comparing all the groups lists under the subheading.

d. Symptoms defined as itching, pain, burning, sores, foul smelling discharge, blood, and pain during sex.

e. Kruskal-Wallis test, shown with median and interquartile range.

Cervicovaginal bacterial communities are not associated with sexually transmitted infections, hormonal contraceptive usage, or sexual behavior

Women with BV have been shown to have a higher incidence and prevalence of STIs (12, 34), be less likely to use hormonal contraceptives (35), and be more likely to engage in high-risk sexual behavior (36). However, we did not find a difference in the prevalence of an active STI or STI symptoms between cervicotypes (**Table 2.1**), though the association with *N. gonorrhea* is difficult to ascertain due to its low prevalence in this group. Additionally, we saw no difference in hormonal contraceptive method, condom use, or reported sexual activity (**Table 2.1**). Thus, there was no evidence that the high ecological diversity in CT4 was due to behavioral, infectious, or exogenous hormonal etiologies.

Large variation in baseline genital immune activation is not explained by STIs

Genital immune activation has been described as an important risk factor for disease of the FGT, such as obstetric complications (37, 38) and HIV acquisition (1, 2). We therefore assessed the cohort's baseline genital immune activation by measuring the concentrations of 17 soluble cytokines in cervicovaginal lavage (CVL) fluid from all participants who had completed at least one mucosal sampling. The cytokine panel included canonical pro-inflammatory cytokines (IL-1 α , IL-1 β , TNF- α), interferons (IFN- γ , IFN- α 2), chemokines (CCL20 [MIP-1 α], CCL4 [MIP-1 β], CCL5 [RANTES], CXCL10 [IP-10], CXCL8 [IL-8]), regulators of inflammation (IL-1 receptor antagonist (IL-1RA), IL-10), differentiation markers (IL-4, IL-12p70), and activation and proliferation markers (soluble CD40L, FLT-3L, IL-2). There was a wide range in CVL pro-inflammatory cytokine levels, with over a 1,000-fold difference in some cytokines (e.g. IL-8 and IL-1 α) among participants. We performed unsupervised clustering to visualize inflammatory patterns (**Figure 2.5A**) and found strong positive correlations (Spearman's $\rho > 0.4$) between 45% of cytokine pairs.

To reduce the dimensionality of the cytokine dataset to a smaller number of covarying components, we performed principal component analysis (PCA) on the normalized cytokine

concentrations. We found that the first principal component (PC1) explained 41% of the variation in genital cytokines; PC1 represented the general presence of inflammatory cytokines, with high and nearly equal loading by all cytokines except for IL-1RA and CXCL10, which had a negligible contribution. Women in the highest or lowest quartile of PC1 had no difference in plasma cytokine levels, indicating that PC1 reflects local, not systemic, immune responses in the genital tract .

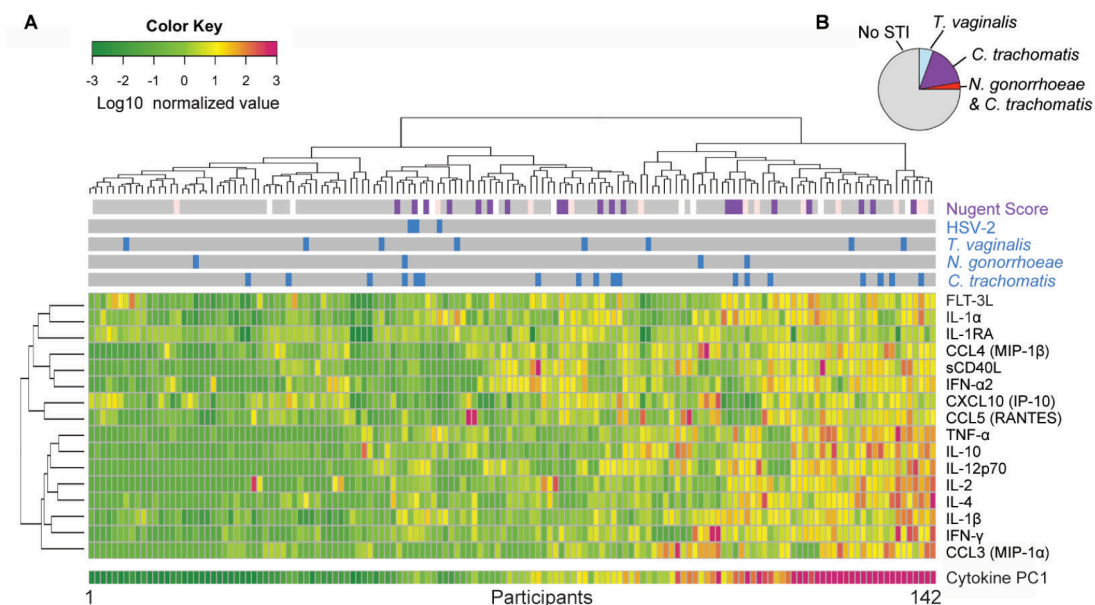


Figure 2.5 Asymptomatic women display a broad range of baseline genital inflammation that is not explained by STIs. (A) Heatmap of cervicovaginal lavage cytokines from 146 women, each represented by a column. Nugent scores and active STIs (blue: *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and/or HSV-2 positive; gray: negative for that STI) are also displayed. Principal component (PC) analysis was performed on the normalized cytokine concentrations and the first PC (PC1) explained 41% of variation. (B) Pie chart of the STI prevalence in women in the highest quartile of inflammation (n=35). See also Figure S3 and Table S1.

We next asked whether women with the highest levels of cytokine inflammation had active STIs. *Chlamydia trachomatis* was the most prevalent active STI in this cohort, with 13% of women testing positive at the first visit. We found that women with *C. trachomatis* had higher inflammation than women without an STI (p=0.0226, Mann-Whitney test) but saw no trend with *N. gonorrhoeae*, consistent with previous studies (39, 40). Of the 35 women in the top quartile of inflammation, 75% did not have a detectable active STI (**Figure 2.5B**). Women in the top

quartile of inflammation also did not have higher rates of HSV-2 seropositivity (55% seropositive in both groups) or more unprotected vaginal recent sex, as determined by detectable prostate-specific antigen in the CVL (2/15 in the high inflammation group, 1/19 in the low inflammation, $p=0.5359$) compared to women in the lowest quartile. Cytokine levels also did not correlate with sexual frequency, condom use, hormonal contraception use, or self-reported STI symptoms (Fisher's exact test with $p=0.05$ significance level). Thus, the cause of elevated genital inflammation in the majority of women remained unexplained.

High-diversity bacterial communities are strongly associated with pro-inflammatory genital cytokines

To assess whether microbial communities might account for non-STI associated genital inflammation, we compared genital pro-inflammatory cytokine levels among CTs, with the hypothesis that *Lactobacillus crispatus* dominant CT1 would have the lowest inflammation due to the beneficial effects attributed to *L. crispatus* (9). To determine aggregate differences in cytokine concentrations, we first examined the value of cytokine PC1 in each CT. There was a highly significant difference across the cervicotypes ($p<0.0001$); between CT1 and CT4 ($p<0.01$); CT2 and CT4 ($p<0.001$), and between CT3 and CT4 ($p<0.05$) (Kruskal-Wallis with post-test, **Figure 2.6A**), even after excluding women with any STIs.

To determine how strongly the presence of CT4, STIs, or BV associated with inflammation (defined by highest quartile of cytokine PC1), we calculated the odds ratios for each and found that only CT4 was significantly associated with cytokine PC1 (OR 4.33, 95% CI: 1.575 – 11.92; $p=0.0046$), though there was a trend for PCR-positive chlamydia (OR 2.380, 95% CI: 0.8497 – 6.666; $p=0.1329$) (**Figure 2.6B**). This indicates that women with CT4 are over four times more likely to have elevated genital pro-inflammatory cytokines than those with CT1-3, and that CT4 is a better predictor of inflammation than STIs or BV.

We next determined which of the cytokines that comprise PC1 were different between CTs. We observed significant increases in IL-1 α , IL-1 β , TNF- α , IFN- γ ($p<0.0001$, **Figure 2.6C-**

F), IL-10 ($p < 0.01$, **Figure 2.6G**), and CXCL8 (IL-8; $p < 0.05$, **Figure 2.6H**), as well as IL-12p70, IL-4, and FLT-3L ($p < 0.01$) in CT4 relative to CT1. Even after excluding women with any STIs, individuals with CT4 still had significantly higher levels of each of these cytokines relative to those with CT1. We observed a less pronounced increase in cytokines in CT3, with significantly higher IFN- γ than CT1, and a trend towards higher TNF- α , IL-8, and IL-10. The genital cervicotypes did not correlate with peripheral CD4+ or CD8+ T cell activation levels. Thus, the highly diverse bacterial communities in CT4 were strongly associated with the presence of multiple pro-inflammatory cytokines, suggesting specific genital bacteria can induce a robust local immune response.

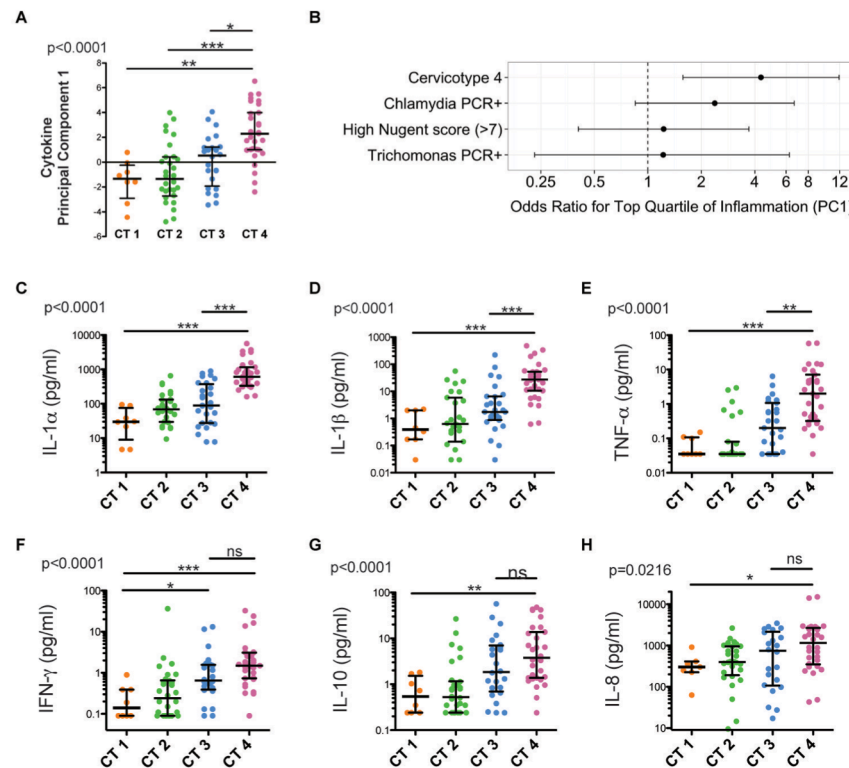


Figure 2.6 Genital pro-inflammatory cytokine levels vary significantly with microbial community structure and most strongly correlate with CT4 bacterial communities. (A) Cytokine PC1 values from women with bacterial communities CT1-4. (B) Odds ratios and 95% confidence intervals representing the likelihood that a woman with cervicotype 4, an active chlamydia infection, bacterial vaginosis (Nugent score >7), or an active *Trichomonas* infection is within the top quartile of pro-inflammatory cytokine levels (as determined by cytokine PC1) versus below the 75th percentile. (C-H) Cervicovaginal lavage IL-1 α , IL-1 β , TNF- α , IFN- γ , IL-10, and CXCL8 (IL-8) concentrations in women with bacterial communities CT1-4. Median and IQR shown. Significance levels were determined by a Kruskal-Wallis test and asterisks denote post-test significance level (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; $n = 94$).

Longitudinal changes in genital microbial communities correlate with pro-inflammatory cytokines

Because of the close association between the genital bacterial communities and inflammation in cross-sectional analysis, we assessed the stability of these factors over time. We performed 16S rRNA gene sequencing and measured soluble cytokines on matched longitudinal cervical swabs and CVLs from all women who had been followed for 6-12 months at the time of analysis. In 58% of sequential time points, women remained in the same CT. Of those who had changes in microbial communities, there was a close association between CTs and IL-1 α , IL-1 β , and TNF- α , which we focused on because they had the strongest associations with CTs in the cross-sectional analysis. Data from two representative individuals who experienced CT changes are shown in **Figure 2.7A**. When we assessed sequential time points for which there was not a CT change, we did not find a significant difference in the canonical pro-inflammatory cytokines ($p>0.05$, one-tailed Wilcoxon matched pairs test; **Figure 2.7B**). However, there were significant increases in IL-1 α ($p=0.033$), IL-1 β ($p=0.042$), and TNF- α ($p=0.030$) in adjacent time points with transitions between a lower and higher CT (one-tailed Wilcoxon matched pairs test; **Figure 2.7C**). Thus, intra-individual changes in genital bacterial communities were tightly linked to inflammatory changes and further support the reciprocal relationship between bacterial communities and genital inflammation.

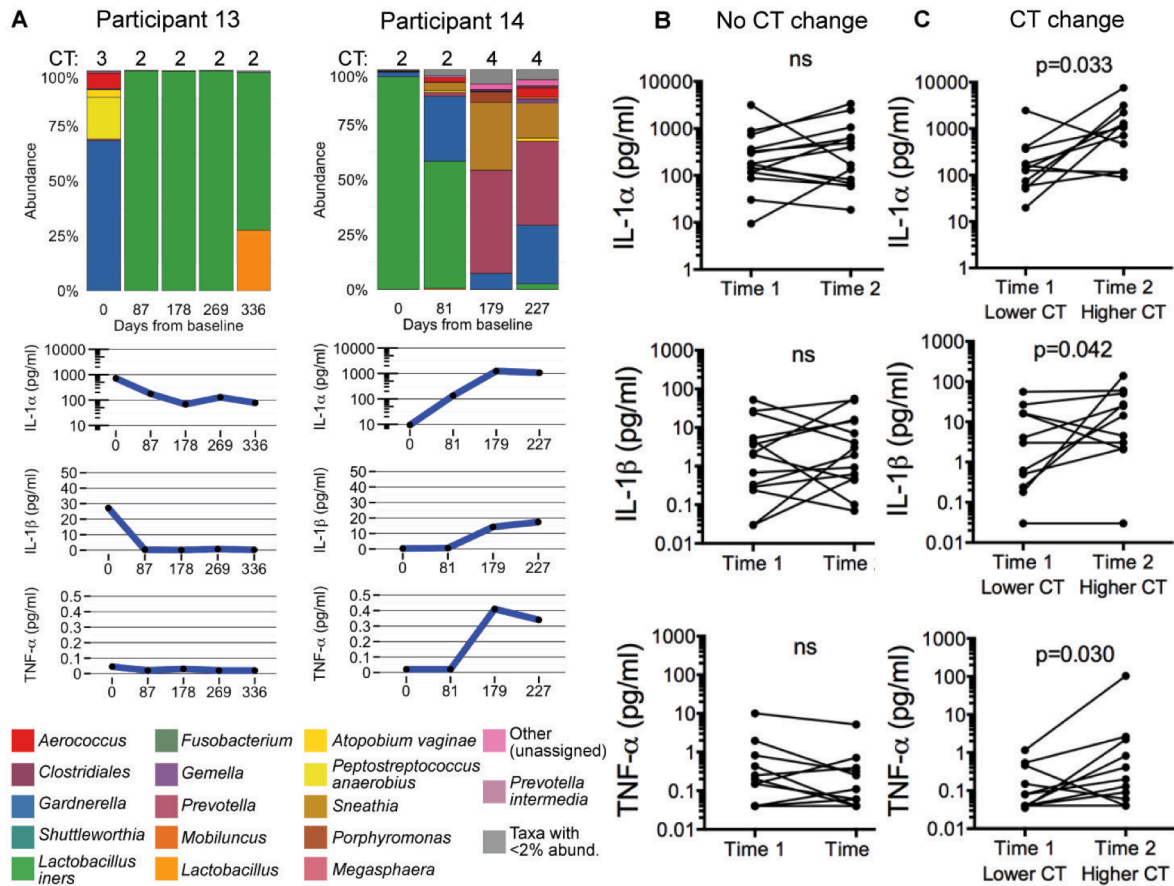


Figure 2.7 Intraindividual longitudinal genital microbiome changes correlate with pro-inflammatory cytokine levels. (A) Cervicovaginal IL-1 α , IL-1 β , and TNF- α concentrations with bacterial taxa identified by 16S rRNA gene sequencing from matched longitudinal cervical swabs and CVLs collected from two representative participants, #13 and #14. (B-C) Cervicovaginal IL-1 α , IL-1 β , and TNF- α concentrations from serial time points with either no change in CT (B) or a change (C) (one-tailed Wilcoxon matched pairs test). The order of time points was standardized such that the lower cervicotype was entered as the first time point and the higher cervicotype was entered as the second time point, regardless of the actual chronological order. Any transition, e.g. from CT1 to CT2 and from CT3 to CT4, was considered to be a CT change.

Women with elevated pro-inflammatory genital cytokines have increased activated cervical HIV target cells

Given the close linkage between CT4 bacteria and genital inflammation, we sought to determine whether pro-inflammatory cytokines would correlate with the presence of activated HIV target cells in the genital tract. Activated CCR5+ CD4+ T cells at the cervical mucosal surface are likely the first susceptible cells

to encounter HIV upon sex with an infected partner (41) and support greater HIV replication upon infection than resting cells (42). We found a significantly higher number of activated (CD38+ HLA-DR+) CCR5+ CD4+ T cells in the endocervical canal of women in the top quintile of cytokine PC1 relative to those in the bottom quintile ($p=0.0361$), but no significant difference in activated CCR5+ CD4+ T cells in the peripheral blood

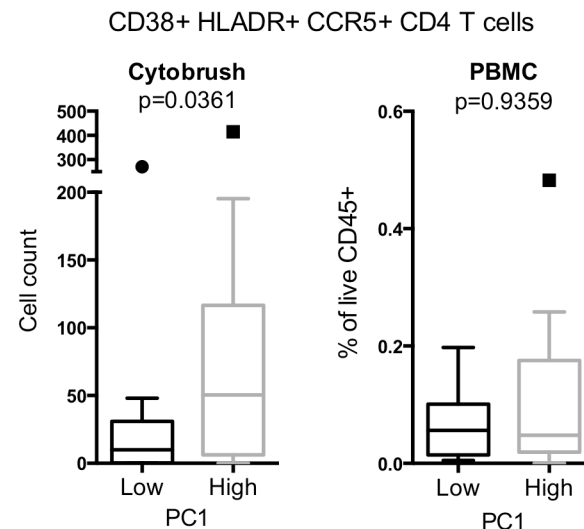


Figure 2.8 Increased activated HIV target cells in the cervix but not the blood of women with elevated genital pro-inflammatory cytokines. Number of cervical and percentage of blood CD38+ HLADR+ CCR5+ CD4+ T cells from women in the highest and lowest quintile of cytokine PC1 (Mann-

(Figure 2.8). Thus, the observed increase in genital HIV target cells supports the link between high diversity microbial communities and HIV acquisition risk.

Trend towards increased HIV acquisition risk amongst women with CT4 bacterial communities

As both genital pro-inflammatory cytokines and BV have been associated with HIV acquisition risk in numerous studies, we hypothesized that women with CT4 bacterial communities have higher rates of HIV acquisition. At the time of analysis, fifteen women had acquired HIV in the FRESH study despite intensive education and counseling. Eight of those women had been enrolled in the study for long enough to have had a baseline pelvic exam prior

to HIV acquisition, and six had multiple pre-infection samples. The pre-infection cervical swabs most proximal to infection were collected between 25-149 days prior to HIV acquisition. Additionally, one participant coincidentally had a pelvic exam during the eclipse phase, just two days before HIV was detectable in her blood and the CVL viral load (VL) was close to the limit of detection with 400 copies/mL. The 15th participant to acquire HIV in the study was started on antiretroviral treatment (ART) just one week after becoming infected.

We compared the pre-infection cervical bacterial communities of the women who acquired HIV to those who remained HIV-negative throughout the study duration. Two of the participants (Acute #8 and #11) had *L. iners* dominant communities, two (Acute #6 and 7) had *Gardnerella* dominance, and the remaining five had CT4 (**Figure 2.9A**). Several bacterial taxa were robustly enriched in women who became infected, including several *Prevotella* taxa, *Sneathia*, *Peptoniphilus*, and *L. iners* (FDR < 0.05, Mann-Whitney test), though their overall abundances were low. Determining HIV acquisition rate differences between CTs was hampered by low sample sizes; comparing the cervicotypes at the extremes, CT4 versus CT1, yielded a hazard ratio of 3.5 that did not reach significance, partially due to the generally low prevalence of CT1 in this cohort (**Figure 2.9B**).

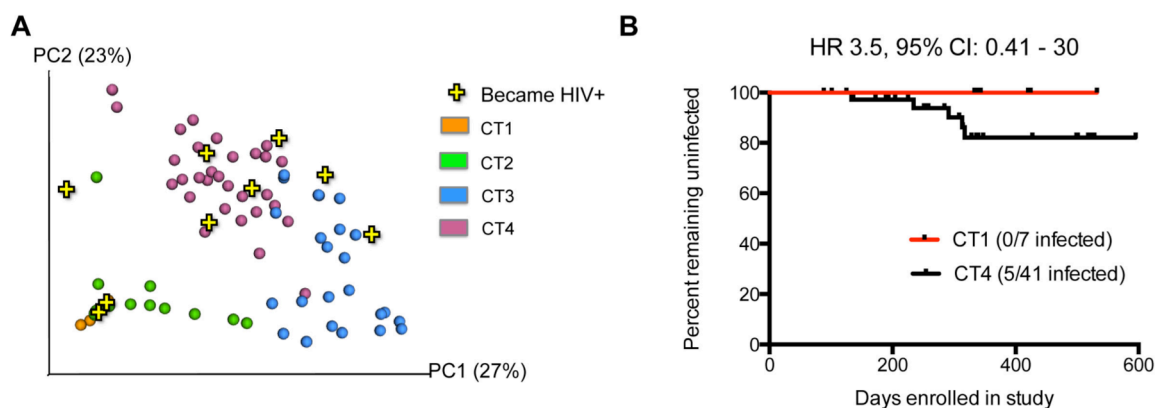


Figure 2.9 (A) Weighted UniFrac PCoA plot representing women who became HIV+ and those who remained HIV negative throughout the study. The most proximal HIV-negative sample collected prior to HIV infection was used. (B) Kaplan-Meier curve representing the HIV acquisition risks of women in CT1 versus CT4. The Hazard Ratio (Mantel-Haenszel) is 3.5, but it is not significant ($p=0.245$ using the Log-Rank test).

Viral dynamics in the genital tract

Following the first positive HIV PCR, plasma viral loads were measured from finger-pricks twice a week for the first five weeks, once a week for the next four months, and once every three months after that. The first positive plasma viral load will herein be referred to as detection or day 0. CVL viral loads were measured at weeks 1, 2, 3, 5, 9, 12, 24, 36, and 48 weeks post-infection, when available. The combination of early detection and high-frequency sampling post-infection affords the unprecedented opportunity to determine viral dynamics in the genital tract. All fifteen participants were found to be infected with HIV before peak viral load was reached in the blood (**Figure 2.10A, B**), with the peak plasma viral load occurring at a median time of 1.5 weeks after detection (**Figure 2.10C**). The participant who received very early ART had undetectable plasma and CVL viral loads by week 4.5. Interestingly, two participants (#8 and #11) consistently had CVL viral loads around the limit of detection despite not receiving ART. Both participants report only having vaginal sex, but not anal sex, within the past 30 days.

Given the paradigm that HIV replicates locally in the genital tract for several days before disseminating systemically, we next asked whether the cervicovaginal lavage VL peaked prior to the plasma VL. We excluded three women for whom there was less than a 2 log difference between the nadir and peak VL, three women for whom a week 2 CVL was not available and thus the peak VL could not be ascertained, and one woman who began ART at week 1. Despite including only 8 women in the analysis, the CVL viral load peak was significantly earlier than in plasma by an average of 5 days ($p=0.03$, **Figure 2.10C**). While cervicovaginal VL significantly correlated with plasma viral load, the low R^2 value indicates that the plasma VL provides some information about the cervicovaginal VL but the prediction is not precise (**Figure 2.10D**). This supports previous findings (43, 44) that the virus appearing in the CVL is not only a transudate from the plasma, but also reflects the distinct microenvironment of the local tissue.

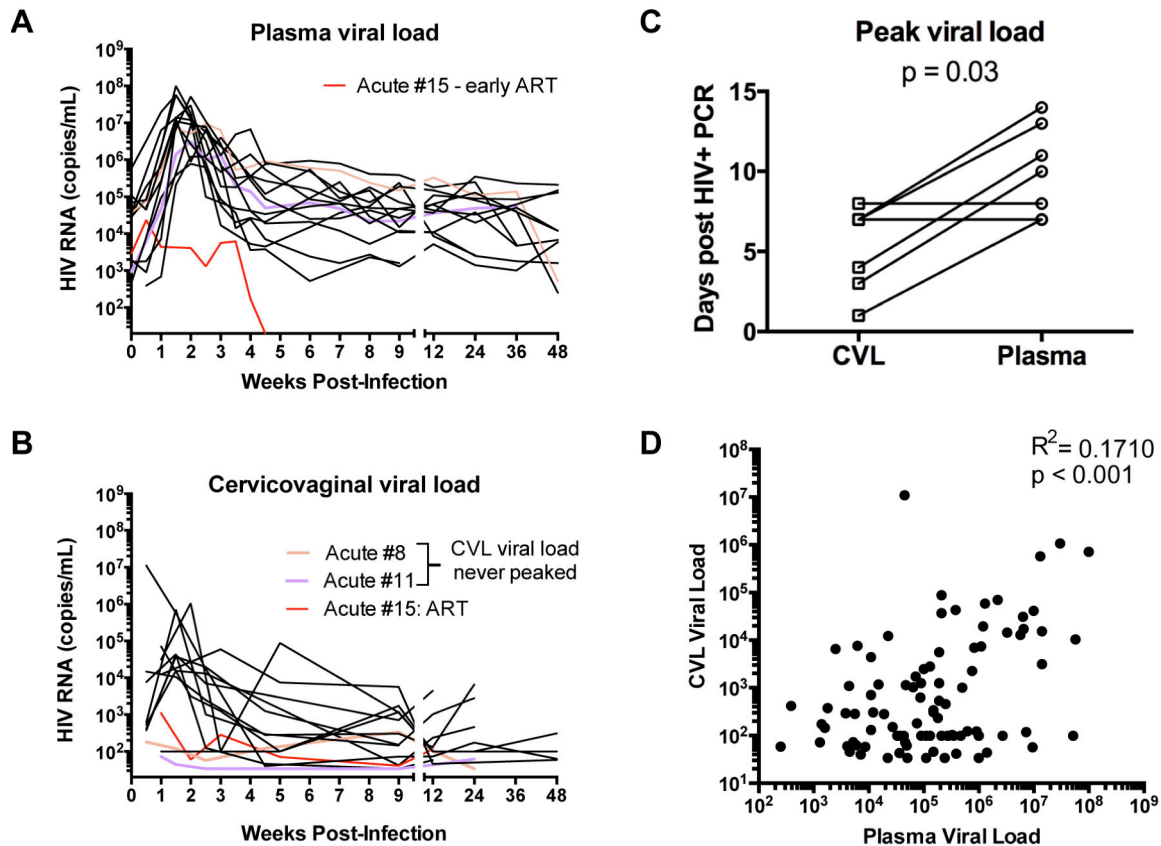


Figure 2.10 Very early of detection of HIV infection reveals divergent viral dynamics in the blood and genital tract. (A) Plasma and (B) cervicovaginal lavage viral loads measured over the first year of HIV infection. (C) Comparison of the time point where peak viral load is achieved in each compartment. There is an earlier viral load peak in the cervicovaginal lavage than plasma (Wilcoxon two-tailed test). The week of peak CVL viral load was only calculated in women for whom there was a 2 log difference between the nadir and peak, which excluded three participants (Acutes #5, #8, #11). Acute #1 and #3 were also excluded because a week 2 CVL was not available for viral load testing. (D) Correlation between plasma and CVL viral loads, with the Pearson r^2 shown.

Distinct bacterial and viral genital cytokine signatures

We next asked whether the inflammatory signature of HIV infection was distinct from the CT4 bacterial signature that we previously defined. We compared pre-infection CVL cytokine levels to those at peak CVL viral load in the eight women where paired samples were available. The virus-specific signature included CXCL10, CCL5, sCD40L, and GM-CSF, which all had significantly higher concentrations at peak genital viral load than before infection and were not associated with particular bacterial communities (**Figure 2.11**). IL-8 and TNF- α were common elements of the viral and bacterial signature, as they were higher in both HIV infection and CT4

(Figure 2.11). Other components of the bacterial signature – IL-1 α , IL-1 β , IL-10, IFN- γ , and IL-12p70 – were not significantly higher at peak viral load, and appear to be specifically associated with CT4. While we are unaware of other studies that measured CVL cytokine levels within the first two weeks of HIV-1 infection, the viral signature is consistent with a previous report in which CVL concentrations of CCL5, sCD40L, and TNF- α , but not GM-CSF or CXCL8, were found to correlate with HIV RNA level in CVLs collected six weeks postinfection (43). Thus, the bacterial and viral cytokine signatures in the genital tract are essentially distinct, reflecting differences in the host responses to these microbes.

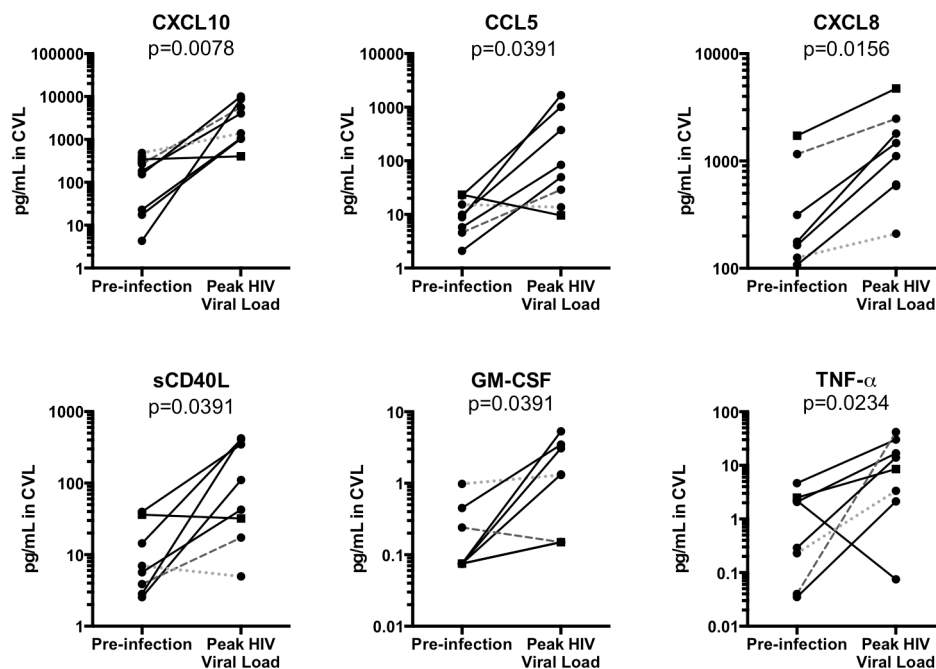


Figure 2.11 CXCL10, CCL5, CXCL8, sCD40L, GM-CSF, and TNF- α are all found at significantly higher concentrations in the genital tract at peak CVL viral load (VL), compared to pre-infection levels. IL-1 α (p=0.64), IL-1 β (p=0.14), IFN- γ (p=0.10), IL-10 (p=0.31), IL-12p70 (p=0.30) were not significantly different (data not shown.) P values were determined using a Wilcoxon matched-pairs signed rank test in 8 women. The two participants with persistently low CVL viral loads are shown with dashed lines (Acute #8) and dotted lines (Acute #11); the timepoint of peak plasma VL was used in lieu of a lack of peak CVL VL.

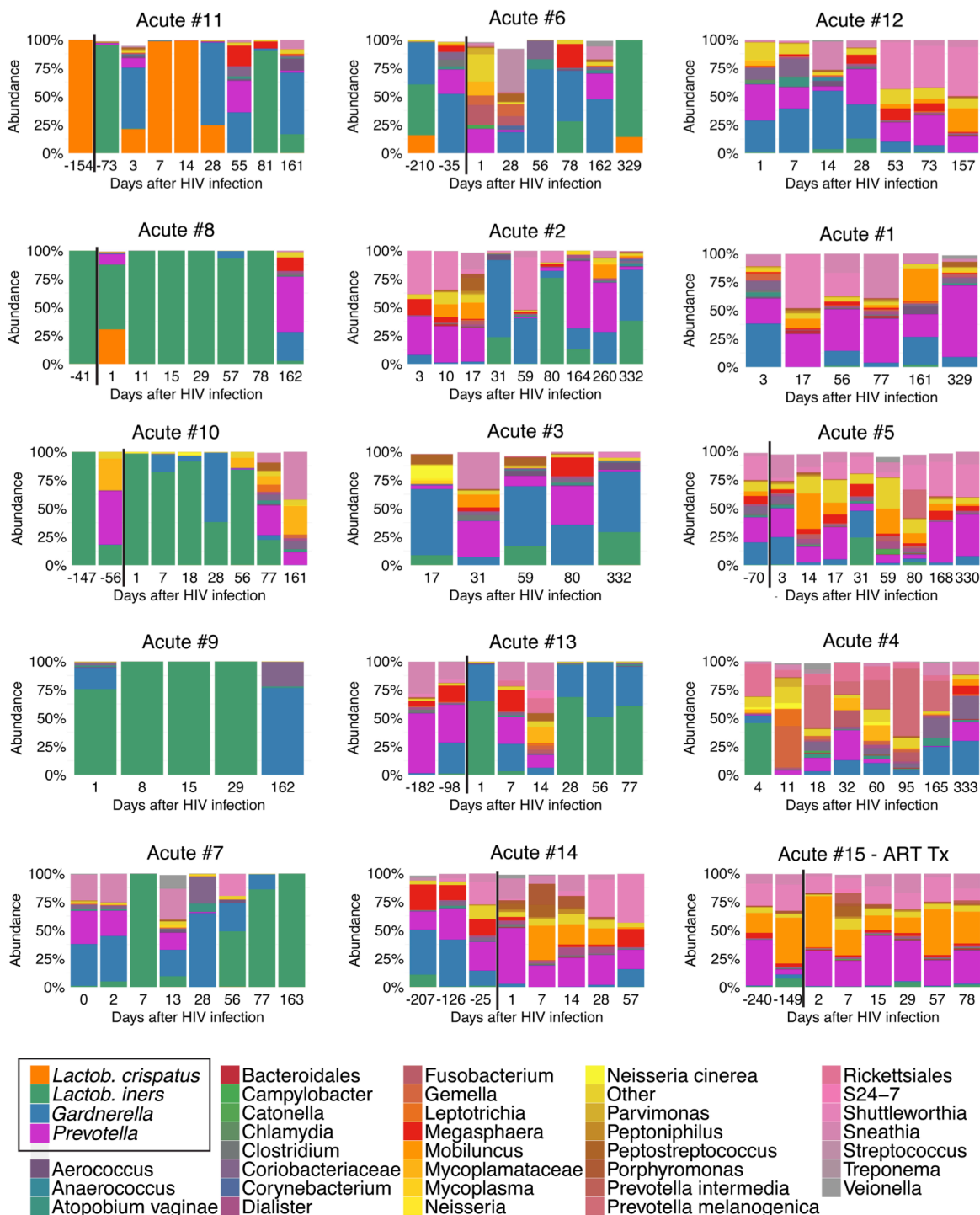


Figure 2.12 16S rRNA gene sequencing analysis from high-frequency cervical sampling in the acute through chronic phases of HIV infection, and prior to infection when available. Day 0 is marked by the first positive HIV RNA results from a finger-prick test. Participants are roughly grouped in columns based on the most abundant cervicotype, with high CT1 abundance in the top left corner. N=15

Stability of the genital bacterial species and communities in acute HIV-1 infection

Finally, we extended the analysis to investigate the composition of cervicovaginal bacterial communities in acute through chronic infection. Given the significant CD4+ T cell depletion in both the genital tract and the peripheral blood that occurs in HIV infection (45), we hypothesized that the cervicovaginal microbiome would become destabilized in HIV infection, with a loss of bacteria that require CD4+ T cell interactions for survival, similar to *B. fragilis* in the gut mucosa (22). To address this question, we performed 16S sequencing on every available cervical swab using an identical procedure used for the HIV-negative swabs. The findings are summarized in **Figure 2.12**.

Proteobacteria was the only bacterial phylum that was significantly different between the acute (first HIV+ swab available) and chronic timepoints and was much higher in the acute phase ($p=0.001$, Wilcoxon paired test with Bonferroni correction; **Figure 2.13**). The Proteobacteria phylum includes both *Neisseria* and, interestingly, *Rickettsia*. An unknown bacteria with 84% sequence identity with several *Rickettsia* species was found at persistently high levels (10-30% abundance) in Acute #4 from weeks 1 through 12 post-infection, and Acute #13

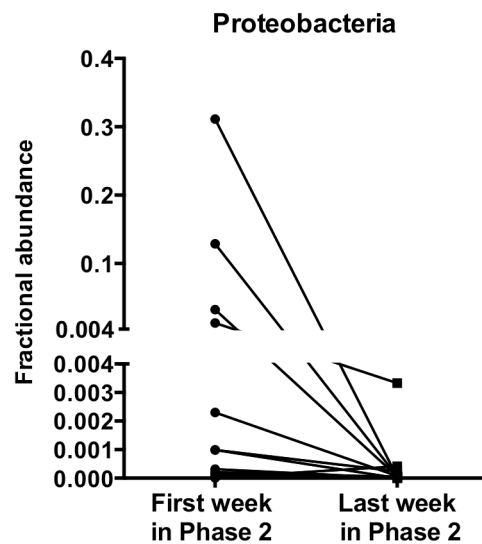


Figure 2.13 Proteobacteria abundance in the acute phase of HIV infection versus the chronic phase.

also had up to a 13% abundance of the same bacteria between weeks 1-3. It was only found in one HIV-negative woman as a singleton. Validating and determining the significance of this likely *Rickettsiales* family member will require further investigation. No other individual species remained significantly different in acute versus chronic infection after multiple correction testing. Notably, Acute #15, who was the first participant to receive ARV therapy within the first week of infection, had a remarkably stable bacterial composition. Despite that observation, we have no

reason to believe that HIV infection alters individual cervicovaginal bacterial members, though we may be missing a small effect due to small sample sizes.

Shifting the focus to communities rather than individual taxa, we compared the stability of the cervicotypes in the absence or presence of HIV. Since the HIV-uninfected women had pelvic exams every three month, but the HIV-positive women had more frequency sampling, in the analysis we only considered samples collected at approximately three month intervals. At these intervals, there was no difference in the likelihood of a participant remaining in the same CT ($p=0.4830$, Fisher's test), arguing against a change in community stability (**Figure 2.14**). However we did find that an HIV-positive woman is less likely to have cervicotype 1 than an HIV-negative woman ($p=0.0465$, Fisher's one-tailed test), confirming previous findings (25). Overall, the cervicovaginal communities remained fairly stable even when faced with an acute viral infection.

A

HIV negative

		To			
		CT1	CT2	CT3	CT4
From	CT1	1	4		
	CT2	2	7	1	2
	CT3		1	1	3
	CT4		1	3	15

B

HIV positive

		To			
		CT1	CT2	CT3	CT4
From	CT1				
	CT2		3	2	4
	CT3		3	1	2
	CT4		1	3	12

Figure 2.14 Enumerating the cervicotype transitions at three-monthly intervals in 13 HIV-negative women and 14 HIV-positive women (excluding Acute #15 who received early ART.)

Discussion

In a cohort of young South African women, we defined four cervicovaginal bacterial community types and demonstrated a strong link between the high-diversity, *Prevotella*-containing CT4 community type and genital inflammation in both cross-sectional and longitudinal analyses. Women with CT4 were over four times more likely to have high pro-inflammatory genital cytokine levels than those with other CTs and on average had higher levels

of genital inflammation than women with BV or STIs. Women who were infected with HIV while enrolled in the study had an enrichment of particular *Prevotella*, *L. iners*, *Sneathia*, and *Peptoniphilus* taxa prior to infection, and there was a trend towards women with CT4 having higher infection rates than those with *L. crispatus* dominant CT1. Finally, the cervicovaginal microbial communities are surprisingly resilient to the perturbation of HIV infection, and do not display a consistent change after infection. Overall, these findings suggest that cervicovaginal bacteria are a major contributor to the basal inflammatory state of the female genital tract and influence HIV acquisition risk.

High prevalence of bacterial communities with low *Lactobacillus* abundance

Only 37% of participants had CT1 or CT2 bacterial communities, clearly indicating that *Lactobacillus* dominance is not typical in this population. This is in contrast to studies in the U.S., in which 90% of white women were found to have *Lactobacillus* dominance (4), but consistent with previous studies that used Gram stain analyses to demonstrate a high prevalence of BV in sub-Saharan African women (46, 47). We report a common occurrence of low *Lactobacillus*, high-diversity communities even in the absence of BV and provide a comprehensive characterization of bacterial taxa using high-throughput sequencing.

The etiology of the low *Lactobacillus* abundance in our cohort remains unexplained. It was not associated with sexual behavior, contraceptive usage or demographic characteristics and we speculate that host genetic factors may play a role. Previous studies have demonstrated a lower frequency of *L. crispatus* and *L. jensenii* and a higher frequency of *Prevotella* in black and Hispanic women compared to white and Asian women in the U.S. (4, 5). The high diversity vaginal communities seen in black American women showed general similarities to those seen in black South African women, specifically with high levels of *Prevotella*, but the prevalence of these high-diversity communities was approximately half of that observed in South Africans. Studies of African cohorts have also demonstrated a higher intestinal *Prevotella* abundance

than in American and European cohorts (48, 49) suggesting that vaginal seeding from the bacteria-rich rectum (50) may also influence the observed differences. Overall, our findings support the growing understanding that *Lactobacillus* dominance is not tantamount to a “healthy” female genital tract microbiome in all women (4), and they highlight the value of bacterial characterization in broader geographic regions.

Strong associations between cervicotypes and cytokines

We found that CT4, and CT3 to a lesser extent, are strongly associated with IL-1 α , IL-1 β , TNF- α , IL-10, IFN- γ , IL-12p70, and CXCL8 (IL-8) concentrations in the CVL, while HIV infection leads to the upregulation of CXCL10, CCL5, sCD40L, GM-CSF, CXCL8, and TNF- α . IL-1 β has consistently been associated with BV, regardless of sample collection method, pregnancy status, and BV diagnosis method (Nugent vs. Amsel criteria). However, differences in TNF- α , IL-10, and IL-8 have been infrequently and inconsistently reported, and IFN- γ and IL-12p70, to our knowledge, were never found to be associated with BV (51-57). Our results elucidate this discrepancy. The traditional Nugent score is calculated by enumerating the number of lactobacilli morphotypes, *Gardnerella* or bacteroides morphotypes, and curved Gram-variable rods (usually *Mobiluncus*) observed on a Gram stain (58). Nearly all studies to date have compared women with BV (either a high or intermediate score) to those without BV. However, we found that the women with highest levels of pro-inflammatory cytokines harbor a particular community type that is *Prevotella*-rich, and *Gardnerella* and *Lactobacillus*-poor. Additionally, the BV score is agnostic to the type of *Lactobacillus* species present, and we saw a clear trend towards lower levels of IL-1 α , IL-1 β , and IFN- γ in women with *L. crispatus* versus *L. iners* in both cross-sectional and longitudinal analyses, a finding that has very recently been demonstrated in a different cohort (59). Thus, the Nugent criteria are too coarse to capture subtle variation in the vaginal microbiota, and molecular characterization of bacterial communities provides greater insight into host-bacterial interactions.

Distinct cytokine patterns associated with bacterial communities and viral infections

The *in vivo* bacterial and viral genital cytokine signatures that were found had a striking resemblance to previously reported *in vitro* studies in which dendritic cells were exposed to Gram-negative bacterial components or viruses (60, 61). For example, one study treated dendritic cells with either *E. coli* or influenza and found that the genes preferentially upregulated in response to influenza infection included those encoding for CXCL10 (strongly), CCL5, and G-CSF, while those upregulated after *E. coli* treatment included IL-1 α , IL-1 β (strongly), IL-12p70, IL-10, and CXCL8 (60). IFN- γ and sCD40L were not among the differentially regulated dendritic cell genes and are likely of T cell origin. The origin and significance of the bacterial cytokine signature is explored further in Chapter 3 of this thesis.

Relative stability of *L. iners*-dominant and CT4-dominant communities in both HIV-negative and HIV-positive women and its implications

While the majority of studies utilize a cross-sectional study design, the vaginal microbiome is increasingly appreciated to be dynamic. Longitudinal studies employing daily (62) or twice-weekly (63) self-collected vaginal swabs in healthy American women found that the most stable community types were those dominated by a single *Lactobacillus* species or the poorly defined community state type IV-A (4). Our analysis was limited by a small cohort and 3-month sampling intervals in the HIV-negative phase. However, the most stable state in both HIV-negative and HIV-positive women appeared to be CT4, with about a 75% likelihood of remaining in CT4 in a 3-month interval. *L. iners*-dominant CT2 was the second most stable community. The overall stability of the cervical microbiome also did not appear to change with HIV infection, as the uninfected women remained in the same CT between serial samples 58% of the time, and the HIV infected women did so 52% of the time. Unfortunately our study did not have the power to determine factors that stabilize or destabilize the microbiome, but prior studies found that the lowest stability was associated with menses (63). The vast majority of

women (>95%) in our study underwent mucosal sampling outside of their menses, thus it is very unlikely to account for the fluctuations that we saw.

The issue of microbial stability becomes particularly important when determining the relationship between the vaginal microbiome and HIV acquisition risk. Reducing the time between the HIV acquisition event and the adjacent previous visit where the genital sampling was performed is critical to drawing accurate conclusions. In large studies, vaginal sampling at intervals less than one month is logistically difficult for both study coordinators and participants. Thus, having the ability to use an archived sample to confidently predict what the vaginal microbial composition was at the time of acquisition would be enormously helpful. Alternatively, if HIV does not effect the vaginal microbiome, studies like ours utilizing high-frequency HIV testing could use the first HIV+ sample as a proxy for the microbial composition at the time of acquisition. Our study suggests, but does not show definitively, that HIV does not significantly alter the vaginal microbiome in very early acute infection. For example, consider Acute #7, who was coincidentally sampled in the eclipse period where her CVL viral load was close to the limit of detection and plasma viral load was negative. She had essentially the same microbial composition two days later, when her plasma viral load had reached a detectable level at 390 copies/mL. Overall, the relative stability of CT4 at a three-month interval is encouraging for previous studies that investigated the relationship between BV and HIV acquisition risk using a similar sampling interval (64, 65).

Impact of cervicovaginal microbiota on HIV acquisition risk

Numerous studies have now shown the two-fold increased risk of HIV-acquisition amongst women with BV (reviewed in (11)), but all of these studies have relied on the Gram stain for bacterial characterization. While our study is not powered to determine the HIV acquisition risk amongst women with CT4 communities, we did see differences in the pre-infection samples of women who became infected compared to those who remained HIV-

negative. Women who became infected had significant enrichment in several CT4 bacteria: *Prevotella*, *Sneathia*, and *Peptoniphilus*. The pro-inflammatory properties of *Prevotella* and *Sneathia* are examined in greater detail in Chapter 3, but larger studies, *in vitro* models, or novel animal models will be required to determine whether these bacteria directly increase HIV acquisition risk.

Materials and Methods

Study cohort and schedule

Participants were recruited through the Females Rising through Education, Support, and Health (FRESH) study, a prospective, 12-month observational study of women 18-23 years old conducted outside of Durban, South Africa. At the time of data analysis, 146 participants had completed at least one mucosal sampling. The study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Durban, South Africa) and the Massachusetts General Hospital Institutional Review Board (2012P001812/MGH; Boston, MA). Informed consent was obtained after the nature and possible consequences of the study were explained. To be eligible for the study, participants had to be female, 18-23 years old, HIV-negative, able to understand the information and consent forms, willing to adhere to study requirements, willing to have HIV testing performed twice-weekly, and willing to have samples stored. Participants could not be pregnant, anemic, or enrolled in any other study.

Twice-weekly volunteers had a finger prick blood draw for HIV RNA testing and every three months had a pelvic exam, peripheral blood draw, and completed an HIV risk questionnaire administered by a counselor.

If a participant was found to be HIV+ by finger-prick, the test was repeated and confirmed the next day. The participant then arranged to have a pelvic exam within the next four days ("week 1") and at weeks 2, 3, 5, 9, 12, 24, 36, and 48 thereafter. Finger-prick HIV RNA testing was performed at weeks 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 12, 24, 36, and 48.

After the protocol amendment was approved, all participants were offered anti-retroviral treatment within the first week of infection detection; unfortunately the approval did not occur until the 15th participant became HIV+.

Sample selection

At the time of analysis, samples were available from 146 study participants who had completed at least one mucosal sampling, 13 participants who had completed at least three samplings, and 15 participants who acquired HIV. Of these 146 participants, we performed 16S sequencing on all subjects who had a complete set of cervical swab, CVL, and cellular phenotyping data (n=94).

Sample collection

The mucosal sample collection involved the collection of ectocervical and midvaginal swabs (Catch-All, Epicentre), a cervicovaginal lavage, and an endocervical cytobrush.

A single nurse performed all the pelvic exams. Ectocervical and midvaginal swabs were immediately placed into sterile cryovials after collection and stored at 4°C for 1-3 hours during transport to the laboratory, followed by long-term storage at -80°C. The first posterior fornix swab was sent to Global Labs (Durban, South Africa) for sexually transmitted infection testing. At the first and fourth pelvic exam visits, the second posterior fornix swab was rolled onto a glass slide, allowed to air dry, and a Gram stain and Nugent scoring (58) were performed by Global Labs.

The cervicovaginal lavage was performed by washing the cervicovaginal walls with 5 mL of sterile saline using a sterile Pasteur pipette. The lavage was kept at 4°C for 1-3 hours during transport to the laboratory, where it was centrifuged at 1700 rpm at 4°C to fractionate the cellular component from the supernatant. The supernatant was aliquoted and stored at -80°C, and subsequently used for cytokine measurements.

Cellular collection was performed by fully inserting a cytobrush (Cytobrush Plus GT, Cooper Surgical) into the endocervical canal and rotating twice exactly 360°. Immediately after collection, the cytobrush was placed into antibiotic supplemented RPMI media with 10% FCS and stored at 4°C for 1-3 hours. In the laboratory, the cells were dislodged from the cytobrush and washed prior to cell surface staining and FACS analysis.

Peripheral blood mononuclear cells were isolated from blood using a standard Ficoll gradient.

BV, sexually transmitted infection (STI), and PSA detection

Vaginal smear Gram staining, Nugent scoring for BV, and STI testing were performed by Global Labs (Durban, South Africa). *Neisseria gonorrhoea* and *Chlamydia trachomatis* were tested using the GeneXpert CT/NG assay (Cepheid). *Trichomonas vaginalis*, HSV-1, and HSV-2 were tested by real-time PCR on a LightCycler (Roche). All positive results for *Trichomonas*, HSV-1, and HSV-2 were retested, and inconsistent results were sent to an outside lab for a third confirmatory test. HSV-2 seropositivity was determined by the HerpeSelect 2 ELISA IgG kit (Focus Diagnostics) on 36 plasma samples. Prostate-specific antigen concentrations were tested using the PSA Quantikine ELISA (R&D Systems) on undiluted cervicovaginal lavage samples. Genital HSV-1 results were negative in all women whose samples were used in this study and thus are not referred to in the manuscript. Women with positive STI results were referred for treatment at an outside facility

Nucleic acid extraction from cervical swabs

We chose to utilize ectocervical swabs for microbial profiling, though we also had vaginal swabs available, because of the closer proximity of the ectocervix to the endocervical canal where the cellular sampling was occurring. The ectocervix is also more relevant to HIV transmission, which is thought to be more likely to occur through the cervical epithelium than the

multi-layered vaginal epithelium (41). Regardless, cervical and vaginal microbial populations are very similar based on data from other studies (66) and our cohort (data not shown).

Swabs were thawed on ice, placed into a solution of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.9, Ambion), 20% sodium dodecyl sulfate (Fisher), Tris-EDTA buffer, and 0.1mm glass beads (BioSpec), and then vigorously rubbed against the walls of the tube. The solution was homogenized using a bead beater for 2 minutes. Following centrifugation at 6,000xg for 3 minutes, the aqueous phase was transferred to a clean tube and an equal volume of phenol:chloroform:isoamyl alcohol was added and mixed by vortexing. Following a second centrifugation at 14,000xg for 5 minutes, the aqueous phase was transferred to a clean tube. Nucleic acid was precipitated with isopropanol and 3M sodium acetate pH 5.5 (Ambion) at -20°C overnight and then centrifuged at maximum speed for 30 minutes at 4°C. The supernatant was discarded and the pellet was washed by adding 0.5mL of 100% ethanol, centrifuging for 15 minutes at 4°C, and discarding the supernatant. The nucleic acid pellet was allowed to dry and was resuspended in 20 µL of molecular grade Tris-EDTA buffer (Promega). The nucleic acid concentration was measured using a Nanodrop (Thermo Scientific).

PCR amplification and sequencing of the V4 region of bacterial 16S rRNA gene

Each cervical nucleic acid sample was diluted to 100 ng/µL using ultra-pure water (MoBio) prior to PCR amplification. PCR amplification was performed using the Phusion High-fidelity polymerase (New England Biolabs) in triplicate, with each well containing 2 µL template DNA. A no-template control was performed for each barcode pair on the same plate. PCR reactions were performed with the following settings: denaturation for 30 s at 98°C, 30 cycles of 98°C for 10 s, 57°C for 30 s, and 72°C for 12 s, with a final extension for 10 m at 72°C. Triplicate PCR reactions for each sample were then combined into a single volume. Amplicons for each sample and a no-template control with the same barcode pair were run on a 1.5% agarose gel to confirm the presence of a single band at roughly 300-350 bp in the amplicon and

absence of a band in the no-template control. Amplicons were quantified by Picogreen (Invitrogen) and equal amounts from each sample were pooled. The amplicon pool was cleaned using an UltraClean PCR Clean-up Kit (MoBio). The library was sequenced with a single 1x300 bp run on an Illumina MiSeq at the Center for Genome Sciences at Washington University in St. Louis using a 10% PhiX spike-in and custom sequencing primers, as described previously (67).

16S sequence quality control and taxonomic classification

Of the 13,964,311 input sequences, 5,975,498 reads passed the quality filtering step (minimum quality score of 30) and were assigned to samples using `split_libraries.py`. Operational taxonomic units (OTUs) were assigned using open-reference picking (97% identity, Greengenes v.13.8) with default parameters except for 0.1% subsampling. The median sequence count/sample was 40,657 with a standard deviation of 14,318. With a rarefaction cut-off of 10,000, one sample containing 1,521 counts was excluded from further analysis. Taxa with fewer than 5 reads were filtered, and the remaining taxa were summarized at the species level.

The principal coordinates plot was generated in EMPeror using the weighted UniFrac (68, 69) method with rarefaction of 2,000 reads per sample. Alpha diversity was calculated using Faith's phylogenetic diversity using the `PD_whole_tree` QIIME command.

Cervicotype determination

"Cervicotypes" were defined as follows: CT1 has a higher percentage of reads assigned by QIIME open-reference OTU picking to the genera *Lactobacillus* (but not *L. iners*) than *L. iners*, *Gardnerella*, or *Prevotella*; CT2 has a higher percentage of reads from *L. iners* than *Lactobacillus*, *Gardnerella*, or *Prevotella*; CT3 has a higher percentage of reads from *Gardnerella* than *Lactobacillus*, *L. iners*, or *Prevotella*; and CT4 is the remaining samples that lack dominance in *Lactobacillus*, *L. iners*, and *Gardnerella*. Shotgun DNA sequencing and oligotyping analyses demonstrated that CT1 was mostly composed of *Lactobacillus crispatus*.

Metagenomic shotgun DNA sequencing and taxonomic classification

Shotgun DNA libraries were made from total nucleic acid isolated from 12 cervical swab samples; the same total nucleic acid was used for 16S amplification. The samples were chosen based on CVL cytokine levels (six with very high inflammation, six with very low inflammation). DNA was isolated from total nucleic acid using an AllPrep DNA/RNA Micro Kit (Qiagen). Methylated DNA was removed using a Microbiome DNA Enrichment kit (New England Biolabs) to enrich for microbial DNA. Microbial DNA was sheared using an S2 sonicator (Covaris) and DNA was sized and quantified using a high-sensitivity DNA Bioanalyzer chip (Agilent). DNA libraries were prepared using a NEBNext DNA Library Construction kit (New England Biolabs), following manufacturer's directions with the double-sided SPRI bead cleanup option. The final library was sequenced on a paired-end 250 bp Illumina MiSeq run. The final library had a median size of 480 bp and was sequenced on a paired-end 250 bp run using the Illumina MiSeq at the Center for Genome Sciences at Washington University in St. Louis. After demultiplexing, each sample had 703,954 - 1,148,817 reads. Quality control filtering and adapter trimming were performed using fastq-mcf (70), with a quality threshold of 25, 0.01% "N" percentage causing cycle removal, and minimum remaining sequence length of 25. The forward and reverse reads of each sample were concatenated prior to MetaPhlAn2 analysis.

Using MetaPhlAn2, we detected the same genera using 16S and WGS methods for every bacteria with an abundance over 1%, with the exception of *Sneathia* and *Shuttleworthia*, which are under-represented in the available marker database due to a paucity of full-genome reference sequences.

Bacterial functional pathways analysis

Reads aligning to the human genome or PhiX were removed from quality controlled and adapter trimmed shotgun DNA sequences. The remaining reads were assigned to KEGG genes

using a translated BLAST search, and HUMAnN (was used to identify the differentially abundant microbial pathways (71).

Oligotyping

Oligotyping analysis (33) was performed on 16S V4 sequencing reads that were assigned by QIIME to the *Lactobacillus* genera but not to a specific species. A total of 233,821 sequences were analyzed; all had Phred quality scores above 30 and were trimmed to 270 bases. Seven base locations of interest (57, 59, 115, 124, 155, 214, and 245) were used to determine oligotypes. The resulting oligotypes had maximum Shannon entropy scores less than 0.2, which is within sequencing noise, indicating that the majority of subtle nucleotide variation was captured. Representative oligotypes were searched in the nucleotide BLAST database; the two most abundant oligotype sequences were:

>Oligotype GCTGCAC

```
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAA
TAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTG
AGTGCAGAAGAGGAGAGTGGAAGTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAG
AACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATG
GGTAGCGAACAGGATTAGAAACCCTAGTAG
```

>Oligotype GCTGCAT

```
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGAAGAA
TAAGTCTGATGTGAAAGCCCTCGGCTTAACCGAGGAACTGCATCGGAAACTGTTTTTCTTG
AGTGCAGAAGAGGAGAGTGGAAGTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAG
AACACCAGTGGCGAAGGCGGCTCTCTGGTCTGCAACTGACGCTGAGGCTCGAAAGCATG
GGTAGTGAACAGGATTAGAAACCCTAGTAG
```

Determination of bacterial taxa enriched in women became HIV+

All HIV-negative and HIV-positive 16S rRNA gene amplicon sequences that were trimmed to a quality score of 30 were combined and open-reference OTU picking with 97% was performed at the 97% OTU level. The OTU table was filtered to remove OTUs with less than 100 reads and OTUs that were not present in at least 10% of samples, then rarefied to 10,000 reads per sample. The `group_significance.py` function was then used to compare the samples from women who remained HIV-negative to the pre-infection samples most proximal to the time of infection using a Mann Whitney U test.

Cytokine measurements in CVLs

Cytokines were measured using a human cytokine/chemokine multiplexed bead assay (Millipore). Cervicovaginal lavages (CVL) were analyzed within six months of collection and underwent a single freeze-thaw cycle. CVLs were thawed on ice, centrifuged for 10 min at 1,000xg and 4°C, and the undiluted supernatant was immediately assayed. GM-CSF, IL-1 β , IL-2, IL-4, IL-6, CXCL8, CXCL10, IL-12p70, IFN- γ , and TNF- α were measured with a high-sensitivity kit. G-CSF, IL-1 α , IL-1RA, CXCL10, CCL3, CCL4, CCL5, sCD40L, FTL-3L, and IFN- α 2 were measured with a regular sensitivity kit. The multiplexed bead assay was performed according to the manufacturer's protocol. A quality control was included with every plate to ensure reproducibility. Cytokine measurements below the limit of detection were assigned to a value of half of the minimum detectable concentration for that cytokine. When generating the heatmap and correlation matrix, measurements for each cytokine were first normalized by taking the log10 of the cytokine concentration, subtracting the mean measurement for that cytokine, and dividing by the standard deviation. The cytokine heatmap was generated using the complete clustering method on the Euclidean distance matrix.

Statistics

Mann-Whitney Wilcoxon test was used to compare continuous data between two groups. For comparisons between more than two groups, Kruskal-Wallis test with post hoc analyses was used. Wilcoxon signed rank was used to compare continuous data between two time-points. Fisher's exact test was used for comparing categorical data among two or more groups. Spearman's correlation coefficients were used to examine associations between variables; only correlations with a *P* value lower than the specified cut-off are displayed. Principal component and cluster analyses were used to obtain summary measures for cytokine/bacteria variables and to identify study subjects with similar cytokine/bacteria profiles. Multivariate analyses using ridge regression (72) were used to identify the correlates of higher cytokine values. *P*-values are two-sided and are not adjusted for multiple testing. The analyses were performed using R Studio and Prism (GraphPad).

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Chapter 3: Understanding the mechanism of host cervicovaginal bacterial sensing in the female genital tract

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Attributions

M.N.A. designed the FACS panel; M.N.A., K.E.D., N.P., and D.S.K. performed F.A.C.S.; M.N.A. and B.A.B. performed nucleic acid isolation; M.S. developed the SCRB-Seq method and performed the sequencing; M.N.A. analyzed the RNA-Seq results; H.S.Y. and R.N.F. designed and performed the *in vitro* bacterial-epithelial cell co-culture experiment; M.N.A. designed and performed the epithelial cell stimulation and inflammasome experiments; M.S.G. provided advice for statistical analyses.

Introduction

The striking cross-sectional and longitudinal association between cervicovaginal pro-inflammatory cytokines and high-diversity, *Lactobacillus*-deficient bacterial communities provokes the question of how these bacteria are sensed by genital innate immune cells. There is a surprising dearth of mechanistic understanding of host-symbiont bacterial interactions in the genital tract, but the gastrointestinal tract and skin serve as valuable paradigms. Symbiotic gut and skin bacteria play critical roles in maintaining local tissue homeostasis through interactions with epithelial cells, macrophages, dendritic cells, Tregs, and Th17 cells (reviewed in (1) and (2)). Toll-like and NOD-like receptors are among several innate sensors used by epithelial cells and antigen presenting cells (APCs) to sense and respond to bacteria at these sites (3-5). The pathways by which particular cervicovaginal bacterial communities elicit pro-inflammatory cytokine production from local APCs and epithelial cells is poorly understood.

Dendritic cells and macrophages are the primary APCs of the cervix and vagina, and they function by directing T cell responses, initiating and resolving inflammation, and maintaining tissue homeostasis (6, 7). The most prevalent APC in the stratified squamous epithelia of the vagina, ectocervix, and ectocervical side of the transformation zone are Langerhans cells that express HLA-DR, CD11c, and Langerin or CD1a (8, 9). Langerhans cells are not present in the columnar epithelium of the endocervix, and endocervical dendritic cell

subsets are poorly defined but many express CD11c, DC-SIGN, and more controversially CD14 (8-13). Macrophages are found in the epithelium of the endocervix and the lamina propria of both the ecto- and endocervix, and express CD68 intracellularly and HLA-DR and CD14 on the cell surface (8). The functional characteristics of APCs in the human vagina are only beginning to be elucidated (13) and are still unknown in the endocervix.

Cervical and vaginal epithelial cells also play a critical role in maintaining the mucosal barrier, interacting with commensal microbiota, and influencing local immune cells including APCs (14, 15). Primary human genital epithelial cells are difficult to study *ex vivo* due to rapid loss of viability, but HPV16/E6E7 immortalized human epithelial cell lines derived from normal vaginal, endocervical, and ectocervical cell lines maintain the morphological and immunological characteristics of primary tissue and provide a simple and reproducible model (16). These cell lines retain the Toll-like receptor expression pattern of the primary tissue (17, 18) and produce cytokines such as CXCL8, CCL5, IL-1 α , and IL-1 β upon stimulation with microbial-associated molecular patterns (MAMPs) (19). Consequently, they have been used to test the inflammatory properties of particular commensal vaginal bacteria, viruses, microbicide products, and STIs (20-23). While these cell lines activate NF- κ B with MAMP stimulation and bacterial co-culture (20, 24), the pathway leading to IL-1 β secretion in these cells is unknown.

Here we elucidate several pathways by which genital antigen presenting cells and epithelial cells sense and respond to individual bacterial species and communities commonly found in young South African women. Additionally, using an epithelial cell–bacterial co-culture model, we demonstrate that CT4 bacteria are capable of directly inducing the secretion of pro-inflammatory cytokines.

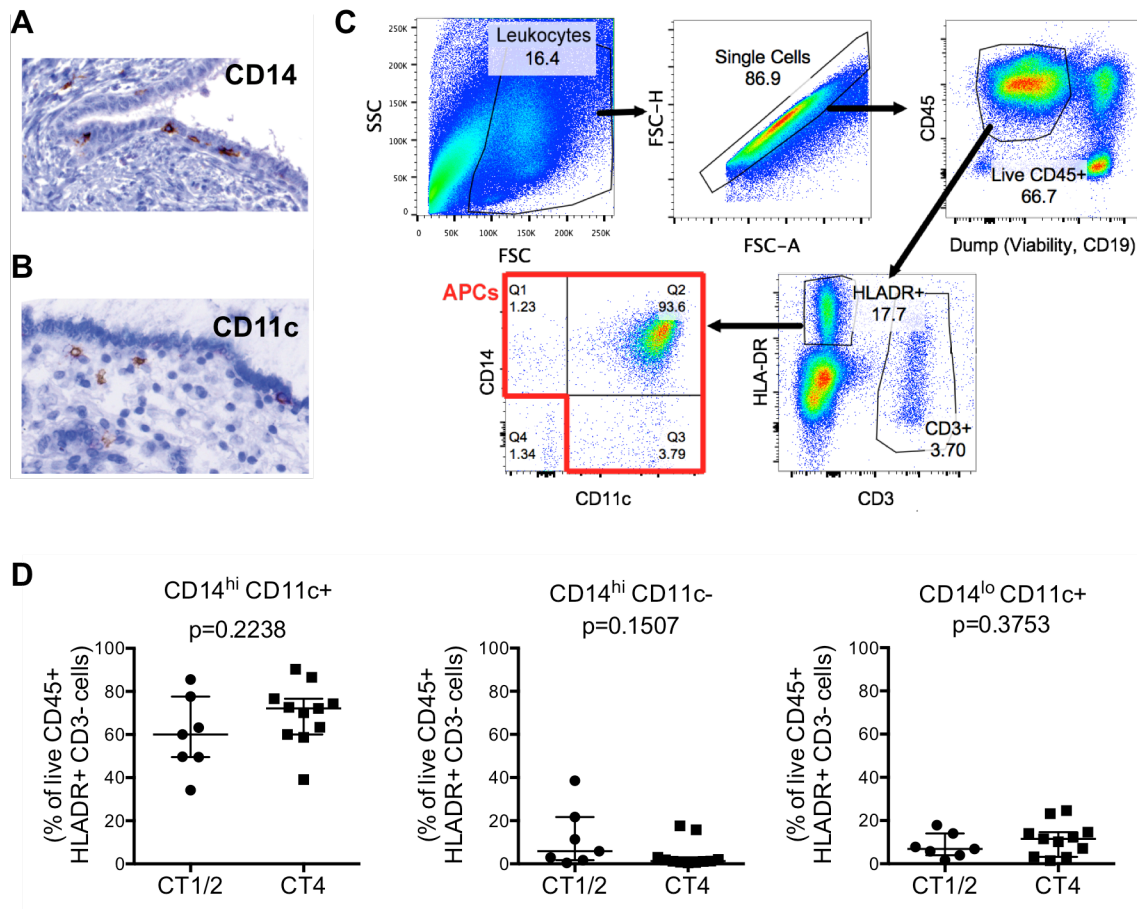


Figure 3.1 Assessment of antigen presenting cell frequency and phenotype in cervical cytobrushes. (A, B) CD14 (A) and CD11c (B) immunohistochemical staining of endocervical tissue. Scale bar: 50 μ m. (C) Gating strategy for flow cytometric analysis. Fluorescence minus one controls were used to determine the positive gates for CD38, HLA-DR, CCR5, and CD25. (D) Comparing APC subset frequency between CT1/2 and CT4.

Results

Genital antigen presenting cells from women with high diversity genital communities are activated by microbial products *in vivo*

Antigen presenting cells (APCs) in the cervix are closely associated with the epithelium (**Figure 3.1A and B**) and sense microbes in conjunction with epithelial cells (25). Because the context in which APCs are stimulated can have important effects on their phenotype and ability to prime adaptive immune cells (1, 14), we performed phenotypic and transcriptional analyses on sorted APCs obtained by cervical cytobrush to understand bacterial sensing *in situ*. Due to the low frequency (10%) of participants with *Lactobacillus crispatus* dominance, we compared APCs from women in CT1 or CT2, who had gram-positive *Lactobacillus* dominance, to those in CT4, additionally excluding anyone with an active STI. Using a basic flow cytometry panel (**Figure 3.1C**), we saw no significant difference in the frequency of cervical APC subsets (CD11c⁺ dendritic cells or CD14⁺ monocyte/macrophages), with most APCs double positive for CD11c and CD14 (**Figure 3.1D**).

However, we did observe significant differences in the APCs between CT1/2 and CT4 by digital gene expression analysis. 35 genes were significantly upregulated and 2 were downregulated in women with *Prevotella* dominant communities relative to those with *Lactobacillus* dominance (false discovery rate [FDR] <0.05; **Figure 3.2**). The genes upregulated in CT4 were enriched in NF- κ B, Toll-like receptor, NOD-like receptor, and TNF- α signaling pathways. Gene set enrichment analysis revealed that 20 of the 35 genes upregulated in CT4 APCs were shared with monocytes treated with a low dose of lipopolysaccharide (LPS, a TLR4 agonist; $q=6.16e-36$) and 19 genes were shared with dendritic cells treated with LPS ($q=1.11e-33$). Ingenuity Pathway Analysis also identified LPS as the most likely upstream regulator to be activated in CT4 APCs ($p=9.47e-26$). These results are consistent with our bacterial pathway analysis using the microbial DNA shotgun sequences, which demonstrated

that LPS biosynthesis (ko00540) was the second most enriched pathway in the high inflammation samples compared to those with low inflammation.

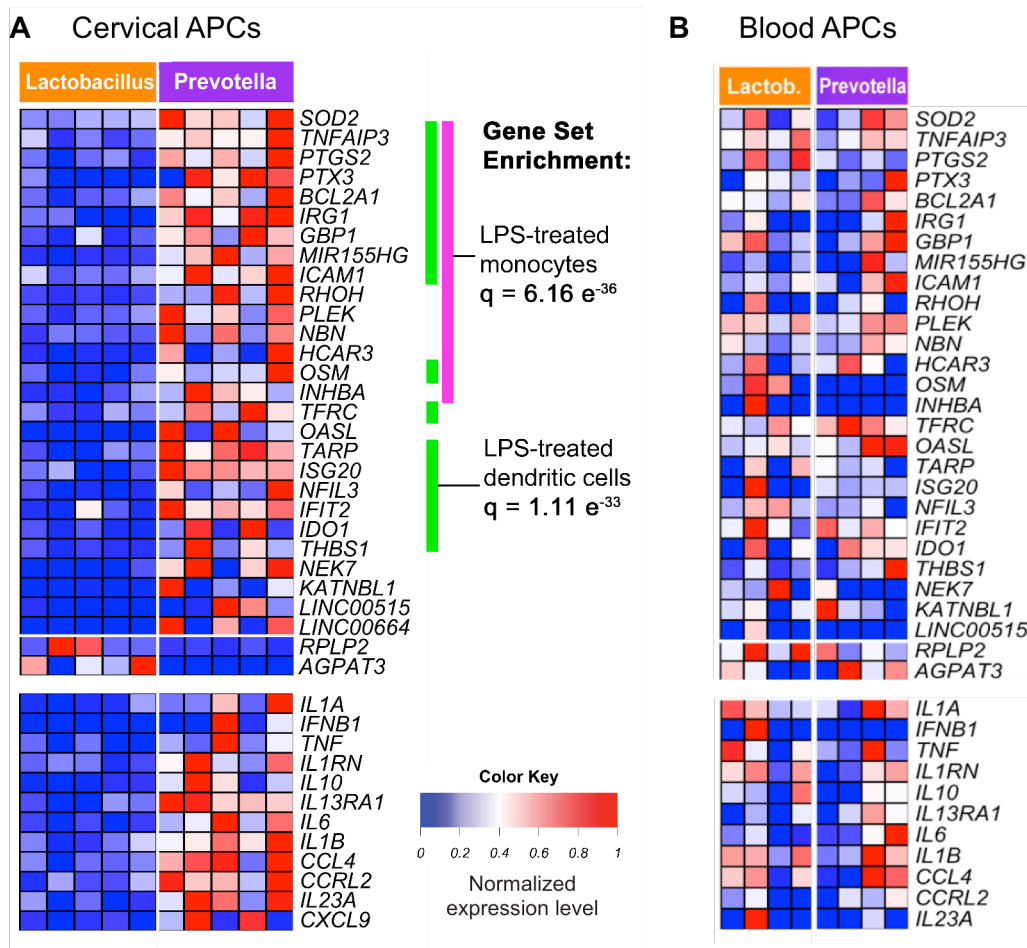


Figure 3.2 Transcriptional profiling suggests that CT4 bacterial products are sensed by cervical antigen presenting cells (APCs), which contribute to genital inflammation by producing a myriad of pro-inflammatory cytokines and critical T cell chemoattractants. (A) Heatmap of genes differentially expressed in cervical APCs from women with *Lactobacillus* dominant communities (>95% abundance) (from left to right: 2 in CT1, 3 in CT2) versus those with high *Prevotella* abundance (>25%) (n=5 in CT4). Women with STIs were excluded. Only genes with a false discovery rate < 0.05 are shown. Gene set enrichment analysis was used to determine statistically significant similarities with annotated gene sets. (B) PBMC APC levels of the same genes differentially regulated in cervical APCs, from the same women (matched PBMCs were not available for 2 women). If no transcripts were present for a given gene, the gene name was omitted from the heatmap.

In addition to LPS, Ingenuity Pathway Analysis also predicted IFN- γ ($p=6.31 \times 10^{-24}$), IL-1 β ($p=1.51 \times 10^{-23}$), and CSF2 (GM-CSF, $p=6.74 \times 10^{-22}$) to be upstream regulators of the CT4 APCs, which is consistent with higher levels of IFN- γ and IL-1 β in the CVLs of women with CT4. Also

consistent with the CVL cytokine measurements, CT4 APCs had significantly higher expression of many pro-inflammatory cytokine genes, such as *IL1A* (5.8-fold), *IL1B* (4.3-fold), *TNF* (6.5-fold), *IL10* (10.6-fold), *IFNB1* (23.4-fold), *IL23A* (7.3-fold), and *IL6* (8.9-fold), compared to CT1/2 APCs (**Figure 3.2A**). Of relevance to cellular recruitment, CT4 APCs also upregulated the chemokines *CCL4* (MIP-1b, a CCR5 ligand, 5.7-fold), *CXCL9* (MIG, a CXCR3 ligand, 9-fold), and *CCL8* (MCP-2, a CCR1 and CCR5 ligand, 6.3-fold). These transcriptional differences were not observed when comparing APCs from the peripheral blood of the same women (**Figure 3.2B**). Thus, the transcriptional profile of cervical APCs indicates that they are responding to a CT4 bacterial product such as LPS and subsequently initiating acute inflammation and lymphocyte recruitment, and that these differences are not a reflection of systemic inflammatory activation.

Identification of bacterial species that cause pro-inflammatory cytokine secretion in a vaginal epithelial cell co-culture model

As CT4 seemed to contain bacterial species with LPS, we shifted our focus from interrogating the effects of communities to identifying the pro-inflammatory effects of particular bacterial taxa. To identify candidate bacteria to test in an *in vitro* system, we asked which bacteria were most highly correlated with inflammation *in vivo*. To select bacterial taxa to use in a multivariate model, we performed univariate Spearman correlations between cytokine PC1 and each taxon found with over 0.1% abundance. The twelve taxa with highly significant correlations ($p \leq 0.005$) were included as predictor variables in the multivariate analysis. Since many taxa were highly correlated, the ridge method was used to correct for multicollinearity and revealed six genera significantly correlated with cytokine PC1: *Fusobacterium*, *Aerococcus*, *Sneathia*, *Gemella*, *Mobiluncus*, and *Prevotella* (**Table 3.1**). Since 16S rRNA amplicon sequencing cannot consistently identify bacteria at the species level, the previously performed whole-genome sequencing analysis was used to identify specific candidate species from these genera.

Table 3.1 Determining which bacterial taxa are most highly correlated with pro-inflammatory cytokines levels.

Ridge regression analysis with cytokine PC1 as the response variable and bacterial abundances as the predictor variables. The bacterial prevalence amongst the 94 subjects is also shown; taxa must have an abundance of at least 1% within a sample to be considered present.

	Prevalence (n=94)	Scaled β coefficient	p value
<i>Fusobacterium</i>	6.4%	5.34	0.0015
<i>Aerococcus</i>	26.6%	5.29	0.0012
<i>Sneathia</i>	40.4%	3.96	0.0168
<i>Gemella</i>	16.0%	3.94	0.0198
<i>Mobiluncus</i>	19.1%	3.63	0.0275
<i>Prevotella</i>	55.3%	3.62	0.0348
<i>Other (unassigned)</i>	47.9%	2.11	0.2034
<i>Shuttleworthia</i>	21.3%	2.05	0.2141
<i>Clostridiales</i>	18.1%	0.76	0.6400
<i>Mycoplasma</i>	4.3%	-0.51	0.7570
<i>Lactobacillus iners</i>	62.8%	0.20	0.9068
<i>Leptotrichiaceae</i>	2.1%	-0.18	0.9120

We used these candidates to test the causative relationship between individual bacterial species and genital inflammation with an *in vitro* epithelial cell co-culture assay. This model has previously been used to demonstrate the pro-inflammatory properties of *Gardnerella vaginalis*, *Atopobium vaginae*, and *Prevotella bivia* (20, 26). We co-cultured immortalized human vaginal epithelial cells with bacterial isolates and measured secreted pro-inflammatory cytokines in the supernatant. Consistent with our *in vivo* findings, *Prevotella amnii*, *Mobiluncus mulieris*, *Sneathia amnii*, and *Sneathia sanguinegens* induced higher levels of IL-1 α , IL-1 β , and IL-8 than *Lactobacillus crispatus* (**Figure 3.3**).

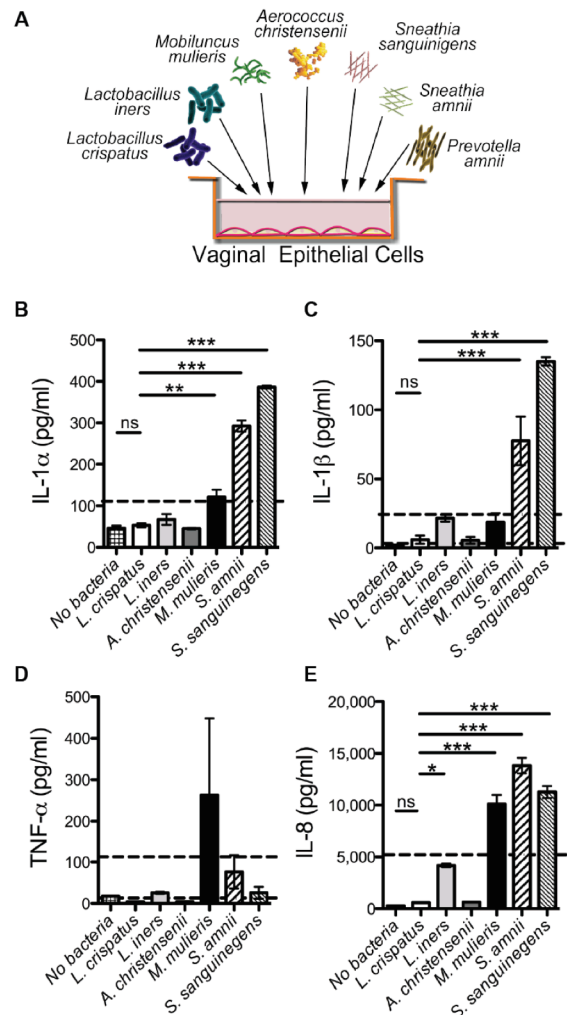


Figure 3.3 Bacterial species highly correlated with pro-inflammatory cytokines *in vivo* also stimulate vaginal epithelial cells to produce the same cytokines *in vitro*. IL-1 α , IL-1 β , TNF- α , and IL-8 secretion by vaginal epithelial cells after *in vitro* application of $15 \log_{10}$ CFU/cm² of each bacterial species. MALP-2 (a TLR 2/4 agonist; 25nM) treatment was used as a positive control (value denoted by dashed line). P values were determined by a Dunnett test, compared to *Lactobacillus crispatus*. Data shown as mean and SEM, with two biological replicates.

Table 3.2. IL-1 α , IL-1 β , TNF- α , and IL-8 induced by *in vitro* application of bacterial species to vaginal epithelial cells. *Prevotella amnii* was only tested at 2 log₁₀ CFU/cm² due to difficulty with growing the bacteria. MALP-2 (25 nM) is used as a positive control. Data shown as mean \pm SEM (performed in duplicate).

IL-1 α (pg/ml)							
log ₁₀ CFU/ cm ²	<i>Lactobacillus</i> <i>crispatus</i>	<i>Lactobacillus</i> <i>iners</i>	<i>Aerococcus</i> <i>christensenii</i>	<i>Mobiluncus</i> <i>mulieris</i>	<i>Prevotella</i> <i>amnii</i>	<i>Sneathia</i> <i>amnii</i>	<i>Sneathia</i> <i>sanguinegens</i>
15	53 \pm 5.0	67 \pm 13	5 \pm 0	121.5 \pm 17.5	n/a	292.5 \pm 13.5	386.5 \pm 3.50
12	51.5 \pm 1.50	56.5 \pm 2.50	2.5 \pm 1.5	66 \pm 4.0	n/a	263 \pm 71.0	252.5 \pm 12.5
8	48 \pm 1.0	46 \pm 6.0	6 \pm 0	49.5 \pm 5.50	n/a	87.5 \pm 13.5	96.5 \pm 2.50
5	56.5 \pm 6.5	43.5 \pm 2.5	0.5 \pm 0.5	46.5 \pm 4.50	n/a	48.5 \pm 1.50	70.5 \pm 10.5
2	52.5 \pm 3.5	45 \pm 3.0	18.5 \pm 16.5	46.5 \pm 1.50	83 \pm 28	47 \pm 2.0	43.5 \pm 1.5
IL-1 β (pg/ml)							
log ₁₀ CFU/ cm ²	<i>Lactobacillus</i> <i>crispatus</i>	<i>Lactobacillus</i> <i>iners</i>	<i>Aerococcus</i> <i>christensenii</i>	<i>Mobiluncus</i> <i>mulieris</i>	<i>Prevotella</i> <i>amnii</i>	<i>Sneathia</i> <i>amnii</i>	<i>Sneathia</i> <i>sanguinegens</i>
15	6.0 \pm 3.0	21.5 \pm 2.5	5.5 \pm 2.5	18.5 \pm 6.5	n/a	77.5 \pm 17.5	135 \pm 3.0
12	3.0 \pm 0.0	8.0 \pm 0.0	4.0 \pm 1.0	5.0 \pm 1.0	n/a	37 \pm 0.0	65 \pm 21.0
8	5.5 \pm 0.5	4.0 \pm 2.0	3.0 \pm 1.0	3.5 \pm 1.5	n/a	16.5 \pm 3.5	20 \pm 3.0
5	4.5 \pm 0.5	3.0 \pm 0.0	5.0 \pm 0.0	3.5 \pm 0.5	n/a	4.0 \pm 1.0	4.5 \pm 0.5
2	4.0 \pm 0.0	2.5 \pm 0.5	3.0 \pm 1.0	2.0 \pm 0.0	5.5 \pm 1.5	3.0 \pm 0.0	3.5 \pm 1.5
TNF- α (pg/ml)							
log ₁₀ CFU/ cm ²	<i>Lactobacillus</i> <i>crispatus</i>	<i>Lactobacillus</i> <i>iners</i>	<i>Aerococcus</i> <i>christensenii</i>	<i>Mobiluncus</i> <i>mulieris</i>	<i>Prevotella</i> <i>amnii</i>	<i>Sneathia</i> <i>amnii</i>	<i>Sneathia</i> <i>sanguinegens</i>
15	4.5 \pm 0.5	25.5 \pm 2.5	5.0 \pm 0.0	263.5 \pm 184.5	n/a	76 \pm 40 156.5 \pm 137.5	26 \pm 14
12	2.5 \pm 0.5	11 \pm 0	2.5 \pm 1.5	53.5 \pm 5.50	n/a		107 \pm 59.0
8	0 \pm 0.5	9.0 \pm 1.0	6.0 \pm 0.0	28.5 \pm 22.5	n/a	10 \pm 4.0	20 \pm 2.0
5	0.5 \pm 0.5	4.0 \pm 1.0	0.5 \pm 0.5	5.5 \pm 1.5	n/a	0 \pm 0	18.5 \pm 6.5
2	0.5 \pm 0.5	19 \pm 12	18.5 \pm 16.5	3 \pm 2.0	69 \pm 11	2.5 \pm 0.5	4.0 \pm 2.0
IL-8 (pg/ml)							
log ₁₀ CFU/ cm ²	<i>Lactobacillus</i> <i>crispatus</i>	<i>Lactobacillus</i> <i>iners</i>	<i>Aerococcus</i> <i>christensenii</i>	<i>Mobiluncus</i> <i>mulieris</i>	<i>Prevotella</i> <i>amnii</i>	<i>Sneathia</i> <i>amnii</i>	<i>Sneathia</i> <i>sanguinegens</i>
15	581 \pm 1.00	4,173 \pm 167	645 \pm 8.00	10,100 \pm 875	n/a	13,800 \pm 744	11,300 \pm 578
12	363.5 \pm 2.50	1,690 \pm 10.0	364 \pm 12.0	2,580 \pm 93.5	n/a	8,560 \pm 505	10,900 \pm 205
8	339.5 \pm 0.500	568 \pm 7.00	314.5 \pm 4.50	481 \pm 43.5	n/a	2,060 \pm 47.0	6,490 \pm 121
5	360.5 \pm 3.50	303 \pm 1.00	315.5 \pm 3.50	302 \pm 17.0	n/a	403 \pm 19	837 \pm 58.5
2	344.5 \pm 28.5	301 \pm 13.0	312.5 \pm 14.5	289 \pm 5.00	1,290 \pm 82.5	275 \pm 4.0	305 \pm 19.0

Prevotella, the genus consistently present in CT4, was able to induce significant cytokine secretion even at an input CFU as low as $2 \log_{10}$ (**Table 3.2**). Additionally, *L. iners* induced moderate IL-8 secretion, consistent with reports that it can have moderate pro-inflammatory activity, while *L. crispatus* and *Aerococcus christensenii* did not elicit any cytokine secretion relative to the absence of bacteria. Low levels of TNF- α were detected in response to these bacteria. These data suggest that epithelial cells sense cervicovaginal bacteria in a species specific manner and contribute to the observed pro-inflammatory cytokines found in women in CT4.

Determining MAMPs that induce inflammasome activation and IL-1 β release from cervical epithelial cells

IL-1 β is the pro-inflammatory cytokine most consistently found to be elevated in cervicovaginal lavages of women with bacterial vaginosis (27-32). Additionally, we found that CVLs from women with CT4 contain an average of 70-times the concentration of IL-1 β than those from CT1 (**Figure 2.4**), and several CT4 bacterial species are capable of inducing IL-1 β secretion from vaginal epithelial cells (**Figure 3.3**). However, surprisingly little is known about the sensing mechanisms leading to IL-1 β release in the genital tract. Given that the secretion of active IL-1 β is tightly regulated by caspase-1 and that we found several bacterial species that induce IL-1 β secretion from epithelial cells (**Table 3.2**), we hypothesized that the inflammasome is activated by BV and CT4 associated bacteria in epithelial cells of the lower female reproductive tract.

We first asked which microbial-associated molecular patterns lead to IL-1 β secretion in cervicovaginal epithelial cells. We utilized immortalized human ectocervical (Ect1/E6E7) and endocervical (End1/E6E7) epithelial cells (16) that were previously shown to express TLR1, -2, -3, -5, -6, -7, and -9, but neither TLR4 nor MD2 (17, 18). End1 and Ect1 cells were treated with poly(I:C) (dsRNA analog, TLR3 and RIG-I agonist), FSL-1 (Pam2CGDPKHPKSF, TLR2/6

agonist), Imiquimod (TLR7 agonist), ODN2006 (TLR9 agonist), and LPS (TLR4 agonist) for 8 h followed by a 50-minute ATP stimulation. The THP-1 human monocytic cell line is known to produce copious amounts of IL-1 β upon stimulation and were included as a positive control (data not shown.) We found significant IL-1 β secretion from Ect1 and End1 cells only in response to ATP primed by poly(I:C), which activates both TLR3 and RIG-I (**Figure 3.4**). End1 cells also secreted IL-1 β in response to poly(dA:dT) (5 μ g/ml), a repetitive synthetic double-stranded DNA sequence that is indirectly sensed by RIG-I (33). The lack of IL-1 β release in response to nigericin (10 μ M), a potassium ionophore, indicates the inability of End1 and Ect1 cells to assemble the NLRP3 inflammasome independent of TLR priming. Staurosporin (1 μ M), a classical inducer of the cell intrinsic apoptotic pathway, was used to measure non-specific IL-1 β released from dying cells.

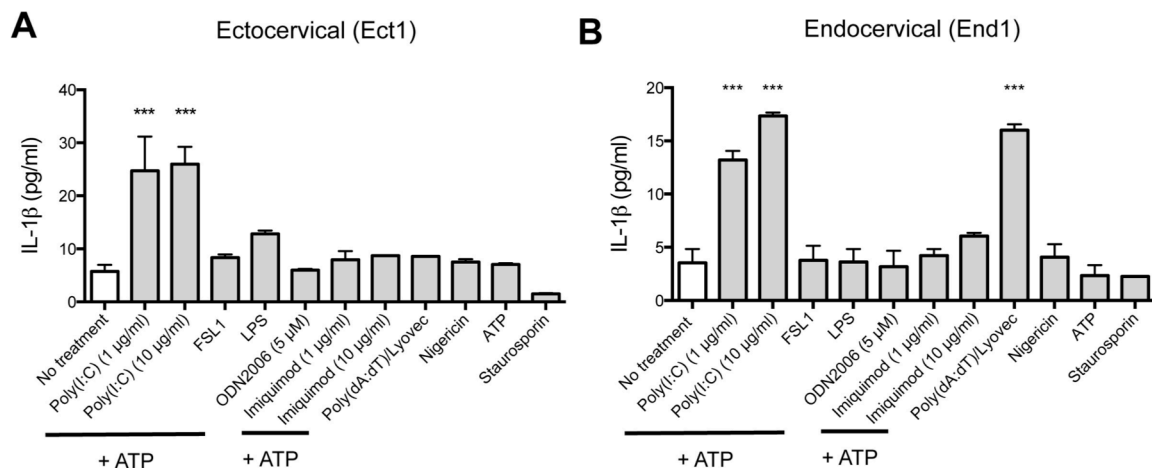


Figure 3.4 IL-1 β secretion is induced by Poly(I:C) in both Ect1 and End1 cells. End1 and Ect1 cells were stimulated with poly(I:C) (TLR3/ RIG-I agonist), FSL-1 (Pam2CGDPPKHPKSF; TLR2/6 agonist), LPS (lipopolysaccharide; TLR4 agonist), ODN2006 (human TLR9 agonist), imiquimod (TLR7 agonist), Poly(dA:dT)/Lyovec (complexed dsDNA; AIM2/RIG-I agonist), nigericin (NLRP3 inducer), ATP (NLRP3 inducer), or staurosporin (classical apoptosis inducer). IL-1 β was detected from cell-free supernatants of A) Ect1 and B) End1 cells using an ELISA (R&D Systems). Assays were performed in duplicate and the data is representative of two independent experiments.

We then asked whether caspase-1 is activated in End1 and Ect1 cells after ATP treatment. Caspase-1 activation was measured using a cell-permeant fluorescent probe that covalently bonds specifically to active caspase-1 via a FAM-YVAD-FMK motif. Necrotic cells

were identified by propidium iodide and excluded. The THP-1 human monocytic cell line has previously been shown to robustly activate caspase-1 when stimulated with PMA and ATP and was included as a positive control. We detected active caspase-1 in End1 cells stimulated by ATP after poly(I:C) priming, and both End1 and Ect1 cells stimulated by ATP after ODN2006 priming, but not by FSL-1, Imiquimod, or LPS (**Figure 3.5**). End1 cells tended to have more caspase-1 activation to a given stimulus compared to Ect1 cells.

As poly(I:C) provided both adequate priming for IL-1 β secretion and caspase-1 activation, we confirmed expression of the poly(I:C) sensors RIG-I and TLR3 in Ect1 and End1 cells. RIG-I is highly expressed in both End1 and Ect1 cells, and upregulated 3-10 fold upon poly(I:C) treatment (**Figure 3.6A,B**). TLR3 was expressed at moderate levels at baseline in both cell types, and upregulated four-fold in End1 cells after poly(I:C). Additionally, both Ect1 and End1 cells weakly expressed NLRP3, the NOD-like receptor that is triggered by ATP, and was upregulated after poly(I:C) stimulation (**Figure 3.6C**). Thus, cervical epithelial cells express several sensors of poly(I:C), and further experiments would be necessary to determine the dominant sensing pathway.

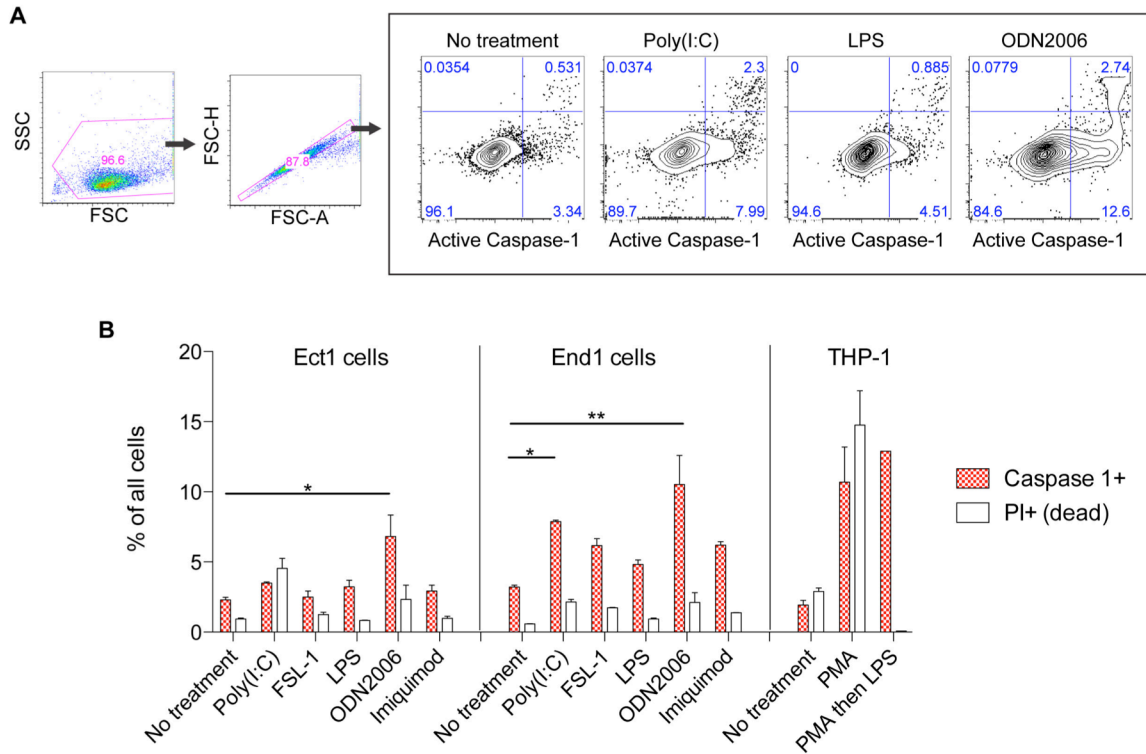


Figure 3.5 Caspase-1 is activated by poly(I:C) in End1 cells and ODN2006 in End1 and Ect1 cells. (A) Representative flow cytometric plots of End1 cells that were treated with a TLR ligand and ATP, then stained with a FAM-YVAD-FMK fluorescent probe to assess caspase-1 activation and propidium iodide to exclude necrotic cells. Necrotic cells were excluded from further caspase-1 analysis. (B) Comparison of Ect1 and End1 caspase-1 activation in response to TLR ligands – poly(I:C) (TLR 3/ RIG-I), FSL-1 (Pam2CGDPKHPKSF) (TLR2/6), LPS (TLR4), Imiquimod (TLR7), or ODN2006 (CpG) (TLR9) – for 8 h followed by ATP stimulation for 50 min. Assays were performed in duplicate and the data is representative of two independent experiments.

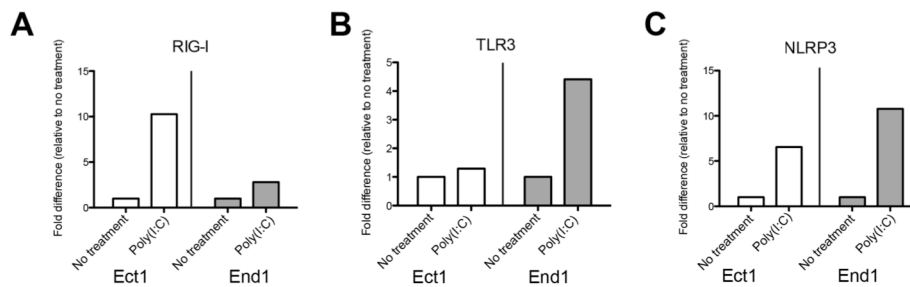


Figure 3.6 TLR3, RIG-I and NLRP3 are expressed at baseline in End1 and Ect1 cells and upregulated by Poly(I:C). End1 and Ect1 cells were stimulated with poly(I:C) (1 ug/ml) for 24 h and qPCR was used to quantify the amount of (A) RIG-I, (B) TLR3, and (C) NLRP3 transcripts relative to an unstimulated control and GAPDH. All three genes were expressed in the unstimulated control and upregulated after poly(I:C) treatment.

Discussion

We show here that CT4 bacterial community members are directly capable of inducing the production of pro-inflammatory cytokines, and provide evidence for two distinct CT4 bacterial sensing mechanisms by cervical APCs and epithelial cells. Cervical APCs specifically appear to respond to LPS via Toll-like receptor 4 signaling, contributing to genital inflammation through NF- κ B activation and recruitment of lymphocytes by chemokine production. This was consistent with the bacterial pathway analysis that demonstrated that LPS biosynthesis was highly enriched in bacterial communities from women with high genital inflammation. In contrast, cervical epithelial cells are insensitive to LPS but both activate caspase-1 and produce IL-1 β in response to a synthetic dsRNA agonist.

Bacterial sensing by genital antigen presenting cells

Transcriptional analyses on APCs isolated from cervical cytobrushes revealed that APCs from women with high *Prevotella* abundance demonstrate a marked response to LPS, as well as to IFN- γ and IL-1 β . This likely reflects both the pathogenic stimulation by gram-negative bacteria and bacterial responses by neighboring cells like epithelial cells, T cells, and natural killer cells. CT4-associated APCs upregulate genes encoding the key cytokines comprising the CT4 cytokine signature: IL-1 α , IL-1 β , TNF, and IL-10. Two long intergenic non-coding RNAs, LINC00515 and LINC00664, were also upregulated in CT4 APCs, and while their function is currently unknown their potential post-transcriptional activities would be interesting to investigate in future studies. Perhaps of greatest importance to HIV acquisition risk, CT4 APCs were also more activated and mature, with higher expression of CD80, ICAM-1, MHC II, and miR-155, and could be contributing to both T cell priming and effector function control (34, 35). This APC activation may be driving the modest but significant T cell infiltration seen in the cervical cytobrushes of women with high pro-inflammatory cytokine levels (**Figure 2.7**), which would have significant implications for HIV acquisition risk.

Direct induction of pro-inflammatory cytokines by CT4 bacteria

Within CT4, we found that *Sneathia sanguinogens*, *Sneathia amnii*, *Mobiluncus mulieris*, and *Prevotella amnii* are capable of inducing the secretion of IL-1 α , IL-1 β , and IL-8 from human vaginal epithelial cells *in vitro*. *Aerococcus christensenii* was highly associated with inflammation *in vivo* but did not induce cytokine secretion *in vitro*, possibly due to the particular bacterial isolate that we tested or because it is merely well-adapted to surviving in inflamed tissue but not a primary driver of inflammation. We did not test *Fusobacterium spp.* due to difficulty with acquiring the proper species, but *Fusobacterium nucleatum* has been shown to be pro-inflammatory and tumorigenic in the gut (36). Overall, several CT4 bacteria are capable of directly inducing pro-inflammatory cytokine production from epithelial cells *in vitro*. It would be worthwhile to extend these co-culture analyses to more complex systems such as those using cervical explants and mixed multilayer cultures of epithelial cells with monocyte-derived macrophages.

IL-1 β production from cervical epithelial cells

Surprisingly, poly(I:C) was the only Toll-like receptor stimulus that provided both adequate priming for IL-1 β secretion and caspase-1 activation in cervical epithelial cells. Poly(I:C) is a synthetic analog of double-stranded RNA and is thus commonly thought of as a virus-associated molecular pattern. However, dsRNA has also recently been shown to be produced by symbiotic bacteria, and is capable of inducing a protective immune response in the gut (37). It is unclear why symbiotic bacteria produce dsRNA, but it may involve CRISPR targeting. Interestingly, the bacterial dsRNA concentration varies amongst bacterial species and growth conditions. Gut lactic-acid producing bacteria have more dsRNA than pathogenic gut bacteria like *Salmonella* and *Listeria*, and the dsRNA concentration increases when bacteria are grown in high NaCl stress conditions (37). While the dsRNA concentration in vaginal

Lactobacilli, *Gardnerella* and *Prevotella spp.* is unknown, we hypothesize that it is highest in *Prevotella spp.* and will pursue this question in future studies.

In conclusion, a plethora of studies have shown associations between bacterial vaginosis and cervicovaginal lavage concentrations of IL-1 β and a handful of other pro-inflammatory cytokines. Here, we make the important mechanistic advancement of individually interrogating the bacterial species comprising the pro-inflammatory bacterial community that we identified in women and determining which are capable of directly causing cytokine production. We determine the bacterial components that are sensed by the local antigen presenting cells and epithelial cells, and propose a novel mechanism, that of dsRNA sensing, for the production of genital pro-inflammatory cytokines.

Materials and Methods

Immunophenotyping of cervical cytobrush samples

Cervical and peripheral blood cells were phenotyped using monoclonal antibodies to the following human proteins in multiparameter FACS analysis: CD4+5 (HI30), CD3 (UCHT1), CD8+ (SK1), HLA-DR (G46-6), CD38 (HIT2), CD25 (2A3), CCR5 (2D7), CD11c (B-ly6), CD14 (M5E2), CD19 (HIB19) from BD Biosciences; and CD4+ (S3.5) from Life Technologies. All antibodies were titrated before use. Viable cells were identified by a LIVE/DEAD violet dead cell stain (Life Technologies). Cells were passed through a 70 μ m filter prior to analysis on the FACS Aria III (BD Biosciences). Rainbow beads (Spherotech) were used to ensure comparable fluorescence measurements between experiments. There were 84 women for whom we had both cervical cellular phenotyping information and CVL cytokine measurements.

Cells were sorted directly into TRIzol (Life Technologies), vortexed vigorously, and stored at -80°C. Data were processed using FlowJo software (Treestar). CD4+ T cells were gated by: FSC vs. SSC, singlets, live CD19-, CD4+5+, CD3+, CD4+ CD8-. Antigen presenting

cells were gated by: FSC vs. SSC, singlets, live CD19-, CD4+5+, HLA-DR+ CD3-, and CD11c+ or CD14+.

Immunohistochemistry

Excess surgical tissue deemed histologically normal by a certified pathologist was acquired through Massachusetts General Hospital (IRB protocol 2010P000632). CD14 (1:200, Clone 7, Abcam) staining was performed on formalin-fixed, paraffin-embedded endocervical tissue sections after antigen retrieval in 1X DIVA Decloaker (Biocare Medical) for 2 minutes. CD11c (1:50, Clone B-ly6, BD) staining was performed on acetone-fixed frozen endocervical tissue sections. After primary antibody incubation, a secondary HRP-labeled goat anti-mouse was applied (EnVision+ System-HRP, Dako). The stain was developed with DAB and enhancement (Dako), followed by a hematoxylin counterstain.

RNA extraction from sorted cells

Briefly, Gen-Elute linear polyacrylamide (Sigma) was added to the RNA in TRIzol, followed by the addition of 0.2 volumes of chloroform and vortexing. After centrifuging at 14,000xg for 5 minutes, the aqueous phase was transferred to a clean tube. The remaining steps were performed as described above (in “Nucleic acid extraction from cervical swabs”), with final nucleic acid reconstitution in 5µL of nuclease-free water. RNA quality and quantity were assessed using the Bioanalyzer (Agilent) and RINs were consistently above 9.

Transcriptional analysis of sorted cells

The RNA-seq libraries were prepared according to the Single Cell RNA Barcoding and Sequencing method originally developed for single cell RNA-seq (SCRB-seq (38)) and adapted to extracted total RNA. Briefly, Poly(A)+ mRNA from extracted total RNA were converted to cDNA decorated with universal adapters, sample-specific barcodes and unique molecular identifiers (UMIs) using a template-switching reverse transcriptase. Decorated cDNA from

multiple samples were then pooled, amplified and prepared for multiplexed sequencing using a modified transposon-based fragmentation approach that enriched for 3' ends and preserved strand information.

All second sequence reads were aligned to a reference database consisting of all human RefSeq mRNA sequences (obtained from the UCSC Genome Browser hg19) and the human hg19 mitochondrial reference sequence using bwa version 0.7.4 with non-default parameter “-l 24”. Digital gene expression (DGE) profiles were then generated by counting, for each microplate well and RefSeq gene, the number of unique UMIs associated with that gene in that well. Python scripts implementing the alignment and DGE derivation are available from the authors upon request.

Digital gene expression values were analyzed using the edgeR package (39). When analyzing the digital gene expression values using EdgeR, counts were normalized by weighted trimmed mean of M-values and significance was tested with a general linearized model.

Bacterial strains and colonization assays

Isolates were obtained from the Culture Collection University of Göteborg (Sweden), the American Type Culture Collection (ATCC, Manassas, VA), and collaborators. The *Lactobacillus crispatus* isolate was originally collected from a vaginal swab from a healthy woman (40). *Lactobacillus iners* (28746), *Aerococcus christensenii* (28831), *Peptostreptococcus* (44095), *Prevotella amnii* (53648), *Sneathia amnii* (64370), and *Sneathia sanguinegens* (41628) were acquired from the Culture Collection University of Göteborg (Sweden). *Mobiluncus mulieris* (35243) was acquired from the American Type Culture Collection (ATCC, Manassas, VA). Bacteria were cultured on Brucella agar under anaerobic conditions and allowed to colonize human vaginal epithelial cells at 2 to 15 log₁₀ CFU/cm² for 24h under anaerobic conditions as previously described (20). IL-1 α , IL-1 β , IL-8, and TNF- α were measured in 24h supernatants

using a Meso Scale Discovery multiplex electrochemiluminescence assay (20). MALP-2 (macrophage-activating lipopeptide-2, a TLR 2/4 agonist; 25nM) was used as a positive control. *Atopobium vaginae*, *Gardnerella vaginalis*, *Prevotella bivia*, and *Lactobacillus gasseri* were not tested due to our prior results indicating that all induce significant inflammatory responses except *L. gasseri* (previously referred to as *L. acidophilus*) (23).

Epithelial cell in vitro stimulation

Ect1/E6E7 and End1/E6E7 cells were grown to 80% confluence, and were stimulated with either poly(I:C) (1 µg/ml of LMW, 1 µg/mL of HMW), FSL-1 (100 ng/ml), LPS (100 ng/ml), ODN2006 (5 µM), or imiquimod (1 µg/ml) for 7.5 hours, then ATP (5 mM) was added for an additional 50 minutes (all reagents from Invivogen). Alternatively, cells were treated with poly(dA:dT)/Lyovec (5 µg/ml, Invivogen), nigericin (10 µM, Invivogen), staurosporin (1 µM, Sigma). As a positive control, THP-1 cells at a concentration of $2-5 \times 10^5$ cells/mL were differentiated into macrophages by an 18-hour phorbol myristate acetate (PMA, 100 ng/ml, Sigma) treatment, treated with LPS (100 ng/ml) for 7.5 hours and then ATP for another 50 minutes. Supernatants were collected and stored at -80C, and then adhered cells were either trypsinized and resuspended in low Ca^{2+} keratinocyte serum-free media, or washed with PBS and lysed in RLT buffer with β-mercaptoethanol (Qiagen).

Caspase-1 activation and IL-1β measurements

Caspase-1 activation was assessed using the FLICA 660 Caspase-1 Assay kit (Immunochemistry), following the manufacturer's protocol except using a 1:120 dilution, which was determined to provide an optimal signal to noise ratio by titration. Dead cells were identified by staining with propidium iodide immediately prior to flow cytometric analysis (4 Laser LSR 2, BD). Soluble IL-1β measurements were performed on cell-free supernatants using an ELISA (R&D Systems).

Epithelial cell RNA extraction and qPCR

RNA was extracted using an RNeasy Micro kit (Qiagen) and cDNA was made using a high capacity cDNA reverse transcription kit (Life Technologies). qPCR was performed using published primers for human RIG-I (F:CTCTGCAGAAAGTGCAAAGC, R:GGCTTGGGATGTGGTCTACT), TLR3 (F: GTGCCAGAACTTCCCATGT, R: CTTCCAATTGCGTGAAAACA), NLRP3 (F: AAAGAGATGAGCCGAAGTGGG, R: TCAATGCTGTCTTCCTGGCA), and GAPDH (all from (41) except TLR3 from (42)) using the Brilliant II SYBR Green qPCR kit per manufacturer instructions.

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Chapter 4: The role of progestin-only injectable contraceptives in increased HIV acquisition risk among South African women

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Attributions

M.N.A. designed the FACS panel and performed the CVL cytokine measurements; M.N.A., K.E.D., N.P., and D.S.K. performed F.A.C.S.; M.N.A. and E.H.B. performed the data analysis and wrote the manuscript with D.S.K.; K.E.D. and A.M. collected behavioral information; M.S.G. provided advice for statistical analyses; A.M. and A.L. performed plasma MPA measurements by mass spectroscopy.

Introduction

Since their introduction in the 1960s, hormonal contraceptives have empowered women to pursue advanced educational and occupational opportunities by preventing unwanted pregnancies while reducing their risk of maternal mortality (1, 2). Hormonal contraceptives can be taken discreetly and, unlike condoms, do not require the cooperation of the male partner. They also come in extremely convenient forms: long-acting injectable progestin-only contraceptives (IPCs) are administered with a single injection every 2-3 months and are over 99% effective. IPCs, including depot medroxyprogesterone acetate (DMPA, marketed as Depo Provera) and norethindrone enanthate (NET-EN, marketed as Nuristerate), are the favored form of contraception in sub-Saharan Africa (3-5). Especially considering all of their advantages, it is most regrettable that IPCs, like other female-controlled contraceptives, not only fail to provide protection against STIs, but also have been found to be associated with a significantly elevated risk of acquiring HIV in some epidemiological studies (6-11).

In areas like sub-Saharan Africa with a high HIV prevalence, the question of whether and how IPCs affect HIV acquisition risk is one of utmost importance. Decisions regarding the withdrawal of IPCs from clinics in these areas must carefully balance the risk of HIV acquisition with the aforementioned risks of unwanted pregnancy, maternal mortality, and limiting women's career opportunities. The epidemiological studies driving these decisions are complicated by behavioral and demographic confounders that may mask a true biological effect of IPCs on

acquisition (12, 13), including that women using IPCs are more likely to be older and in stable relationships and less likely to use condoms than women not using a family planning method. Furthermore, they cannot establish whether association with increased HIV acquisition risk – as high as 4-fold (14) but on average 1.4 fold – represents a causal effect of the contraceptive or is simply associated with contraceptive use because of sexual behaviors that may both inform contraceptive choice and HIV acquisition risk. While progestins have been shown to have a multitude of effects on immune function *in vitro* (15-19), it is still unclear how these effects manifest *in vivo* to modulate HIV acquisition risk.

Understanding how IPCs biologically modify the female genital tract (FGT) *in vivo* can provide a mechanism by which IPCs could lead to increased HIV acquisition risk. We therefore assessed alterations in the FGT immune environment in the context of IPC use in a cohort of young women at high risk of acquiring HIV, attempting to draw a biological link between IPC use and the epidemiological association with increased HIV acquisition risk.

Results

Contraceptive usage amongst FRESH study participants

We first determined which contraceptive methods were utilized by the FRESH participants. Consistent with previous studies, of the 74% of participants who reported having vaginal sex in the past 30 days, only 39% reported always using a condom. Of the 41% of women in the cohort who used some form of non-condom contraception, herein referred to as family planning, 87% of participants were using an IPC (**Figure 4.1**). Three times as many women used DMPA than Nuristerate. The

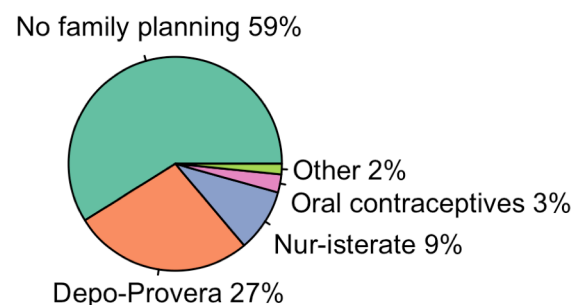


Figure 4.1 Family planning methods utilized by the FRESH cohort (n=253).

remaining women on family planning used oral contraceptive pills (7% of family planning users), an intrauterine device, or tubal ligation.

IPC use was self-reported but verified using two methods: by measuring plasma hormone levels in a randomly selected subset of 222 women, and by directly measuring plasma medroxyprogesterone levels in a subset of women. As expected, women using an IPC had significantly suppressed progesterone ($p<0.001$) and estradiol ($p<0.0001$) concentrations (**Figure 4.2**), with only two out of 84 IPC users demonstrating plasma progesterone levels within the expected range for a woman in the luteal phase. There were no differences in progesterone or estradiol levels amongst women using DMPA and Nuristerate. Of the women who were not using family planning, 129 were sampled during the follicular phase, as indicated by a progesterone concentration of 0.3 ng/mL or less, and 77 were sampled during the luteal phase, with a progesterone concentration at 1.2 ng/mL or more. Thus, we confirm that women using an IPC have suppressed estradiol and progesterone production, and self-reports appear to accurately reflect hormonal contraceptive usage.

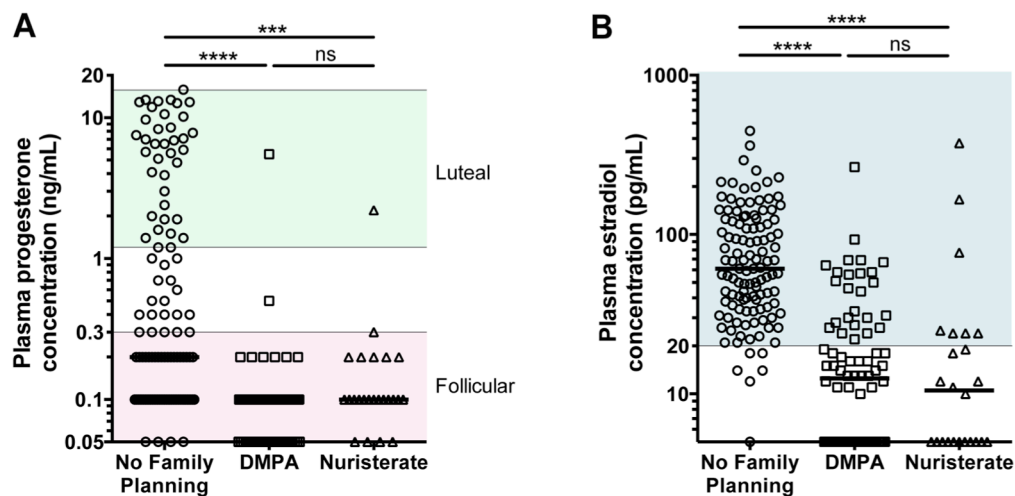


Figure 4.2 Plasma progesterone and estradiol concentrations in women not using family planning (n=114), using DMPA (n=84), or using Nuristerate (n=24). (A) Expected progesterone ranges for the follicular and luteal phases of menstruating women are indicated in pink and green shading, respectively. (B) Expected estradiol ranges for normally menstruating women are indicated by blue shading. Medians are shown with a horizontal line. P values determined by Kruskal-Wallis test with a Dunn's post test. *** indicates $p<0.001$; **** indicates $p<0.0001$. Single timepoints were included for 173 women and multiple timepoints for 49 women.

The second verification that we performed was to use mass spectroscopy to directly measure the active compound of DMPA, called medroxyprogesterone acetate (MPA), in the plasma of a randomly selected subset of women who self-reported using DMPA, Nuristerate, or not using family planning. Of the women who reported using DMPA, 41 out of 44 (93%) had detectable MPA levels, consistent with using DMPA. Two of three without detectable MPA reported receiving their first injection three months before, thus the drug levels may have diminished by the time of sampling. One out of nine women who reported using Nuristerate had detectable DMPA, indicating that the participant misreported her contraceptive use. Finally, one out of eight women who denied using family planning had detectable DMPA. Thus, self-reports provide a fairly accurate account of contraceptive usage.

Next we asked whether there were differences in demographic or behavioral risk factors between women using IPCs compared to women not using family planning. In univariate analysis, statistically significant differences were observed in the age of the participant (IPC users were one year older on average, $p=0.0011$, Mann-Whitney test), the age difference between the participant and their current sexual partners (the difference was one year greater for IPC users, $p=0.0124$, Mann-Whitney test), and the number of vaginal sex episodes in the past seven days (IPC users reported one more sexual act, $p=0.0022$, Mann-Whitney test) (**Table 4.1**). No differences were seen in condom use, anal sex frequency, casual partner count, STI frequency, or bacterial vaginosis frequency. Thus, despite the narrow age range dictated by the study enrollment criteria, women using IPCs tended to be slightly older with slightly older partners and more sexually activity.

Table 4.1 Demographic, sexual behavior and sexual health characteristics of FRESH participants who use injectable progestin-only contraceptive (IPCs) versus those who do not use a non-condom family planning method. Data are presented as n/N (%) or median (IQR). *P-values are determined by Fisher's exact test for categorical data and by Mann-Whitney for continuous data.

	Overall (N = 262)	IPC (N = 95)	No Family Planning (N = 141)	p-value*
Sexual demographics				
Age of participant				
Mean	20.8	21.2	20.5	
Median (range)	21 (18-24)	21 (18-24)	20 (18-24)	p=0.0011
Age difference between participant and current sexual partner				
Mean	3.5	4	3.2	
Median (range)	3 (-4, 17)	4 (-2, 14)	3, (-4, 17)	p=0.0124
Sexual behavior				
Condom use in past 30 days-- no./total no. (%)				
Always	49/235 (20.8)	20/93 (21.5)	27/130 (20.8)	
Sometimes	78/235 (33.2)	33/93 (35.5)	40/130 (30.8)	
Never	47/235 (20.0)	23/93 (24.7)	20/130 (15.4)	p=0.5526
Not applicable (no sex)	61/235 (26.0)	17/93 (18.3)	43/130 (33.1)	
Vaginal sex episodes in past 7 days				
Mean	0.54	0.67	0.45	
Median (range)	0 (0, 4)	1 (0, 3)	0 (0, 4)	p=0.0022
Vaginal sex episodes in past 30 days				
Mean	2.3	2.6	2.1	
Median (range)	2 (0, 18)	2 (0, 18)	2 (0, 12)	p=0.0733
Anal sex in past 30 days -- no./total no. (%)	5/237 (2.0)	1/92 (1.1)	3/133 (2.2)	p=0.6466
Casual or new sex partner in past 30 days	2/227 (0.8)	1/88 (1.1)	1/128 (0.7)	p=1.000
Sexual health-- no./ total no. (%)				
<i>Neisseria gonorrhea</i>	12/226 (5.3)	3/92 (3.3)	8/122 (6.6)	p=0.3581
<i>Chlamydia trachomatis</i>	25/223 (11.2)	8/92 (8.7)	17/119 (14.3)	p=0.3902
<i>Mycoplasma genitalium</i>	18/226 (8.0)	4/92 (4.3)	14/122 (11.5)	p=0.0817
<i>Trichomonas vaginalis</i>	9/226 (4.0)	3/92 (3.3)	5/122 (4.1)	p=1.000
HSV-1	1/226 (0.4)	0/92 (0)	1/122 (0.8)	p=1.000
HSV-2	6/226 (2.6)	2/92 (2.2)	4/122 (3.3)	p=0.7017
Bacterial vaginosis (score ≥7)	39/220 (17.7)	12/89 (13.5)	26/119 (21.8)	p=0.1479

Increased HIV acquisition risk amongst women using IPCs

We next asked whether women using IPCs had higher rates of HIV acquisition. Despite intensive counseling and education, the HIV incidence in the cohort is approximately 7 per 100 person-years, corresponding to 15 women becoming infected by the time of analysis. Strikingly, in our study IPC users had a 5.5-fold higher risk of becoming infected with HIV than women not using family planning (p=0.0031, 95% CI: 1.733 – 16.80, Log-rank test) (**Figure 4.3A**). IPC use

remained significant even after the covariates of the number of sexual acts in the past seven days and the age difference between the participant and the partner were incorporated into a Cox proportional hazards model (HR: 4.87, 95% CI: 1.290 - 18.403, $p=0.0195$). When IPCs were considered individually, Nuristerate users were at greater risk of HIV acquisition compared to those not on family planning ($p<0.0001$, HR: 13.2, 95% CI: 19.58 to 1584, Log-rank test) than DMPA users ($p=0.0730$, HR: 3.861, 95% CI: 0.8821-16.90, Log-rank test) (**Figure 4.3B**). Thus, we found that IPC users were five times more likely to acquire HIV than women not on family planning, and that Nuristerate users were at greater risk than DMPA users.

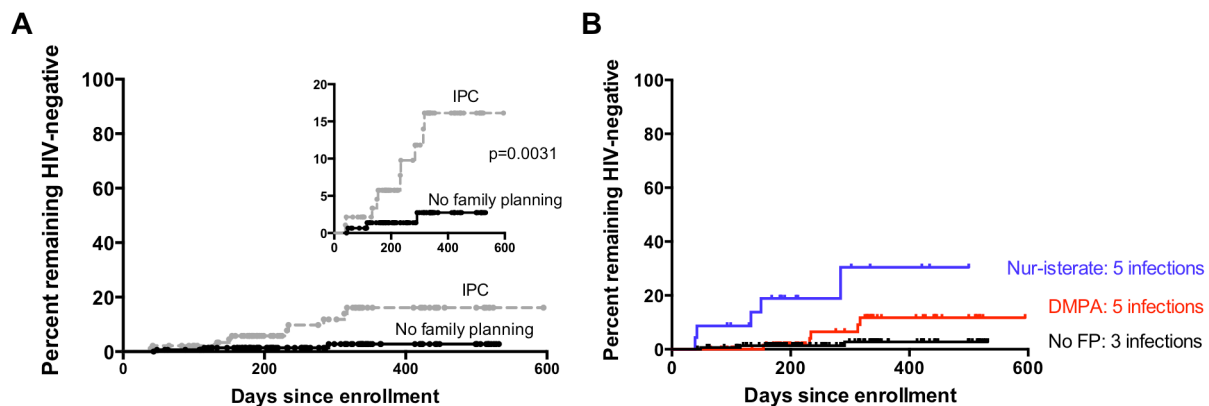


Figure 4.3 Kaplan-Meier curve comparing HIV acquisition rates amongst (A) all IPC users (B) and Nuristerate and DMPA users separately, compared to those not using a family planning method. The P value was determined by a log-rank test.

IPC users have a higher frequency of activated HIV target cells in the endocervix

We next sought to determine immunological correlates of IPC use that may explain the association of IPCs with HIV infection. An important determinant of HIV acquisition risk is the frequency of HIV target cells at the mucosal site of exposure, which in women is thought to primarily be CCR5+ CD4+ T cells in the cervix (20-22). To test the hypothesis that IPCs increase target cell frequency at the superficial layers of the cervix, we performed FACS analysis on freshly collected matched cervical cells and peripheral blood. Because IPCs affect

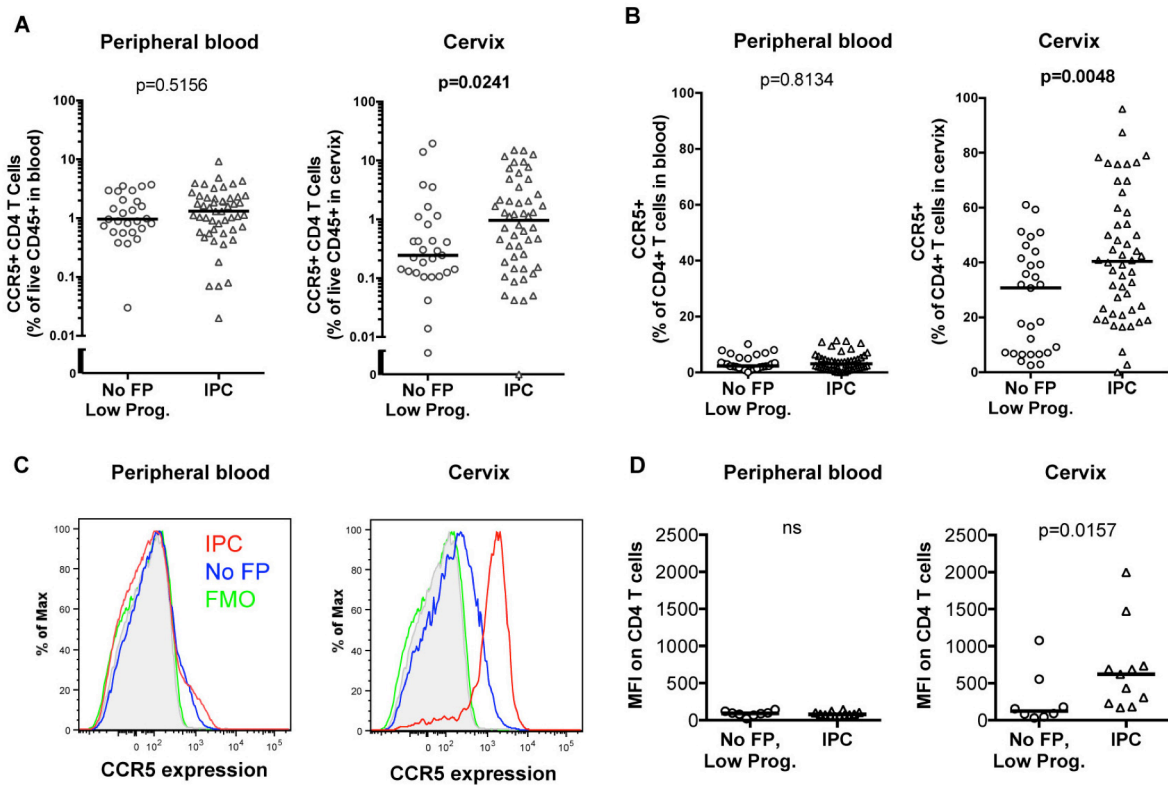


Figure 4.4 IPC use is associated with increased cervical HIV target cell frequency. Controlling for low endogenous progesterone level, injectable contraceptive use correlates with **(A)** a higher frequency of HIV target cells (defined as CD4+CCR5+ T cells) as a percentage of live CD4+5+ immune cells, **(B)** a higher proportion of CD4+ T cells expressing CCR5, and **(C,D)** higher CCR5 expression on CD4+ T cells from the cervix but not the blood. Representative flow cytometry plots of CCR5 expression on CD4+ T cells are shown in **(C)**, where the red histogram indicates a woman using an IPC, blue indicates a woman who is not using family planning (FP), and green with gray shading represents the fluorescence-minus-one control of background fluorescence. Only cytobrushes with over 500 live CD4+5+ cells were included in the analysis.

endogenous hormone levels (**Figure 4.2**) and endogenous progesterone may have its own effects on immune function, we controlled for progesterone levels below 0.3 ng/mL in the analysis, thus comparing women on IPCs to those in the follicular phase of the normal menstrual cycle. Additionally, we assessed DMPA and Nuristerate together as IPCs to ensure adequate sample sizes to uncover differences. We found significantly higher frequencies of HIV target cells as a percentage of live CD4+5+ cells in cervixes of women using IPCs than those not using family planning ($p=0.0241$), though we did not find differences in the blood (**Figure 4.4A**). There was no difference in the number of live cervical CD4+5+ cells recovered from the cytobrushes in the two groups. Additionally, the cervical CD4+ T cells of IPC users had higher CCR5 expression on the cell surface, as measured by the proportion of CD4+ T cells expressing CCR5 ($p=0.0048$, **Figure 4.4B**) and the median fluorescent intensity of CCR5 staining ($p=0.0157$, **Figure 4.4D**). The proportion of CD4+ T cells expressing CCR5 is on average 10-fold lower in the peripheral blood than the cervix, and we did not detect significant differences in the CCR5 expression on peripheral blood CD4+ T cells between women using an IPC and in the follicular phase (**Figures 4.4B-D**). Thus, we found that IPC users have higher CCR5 expression on cervical CD4+ T cells, thereby increasing the number of HIV-susceptible target cells at the mucosal site of HIV exposure.

Because activated target cells produce more viral particles per cell than resting target cells (23, 24), which may accelerate viral dissemination (25), we next assessed target cell activation using three well-established cell surface markers: the IL-2R α chain (CD25), HLA-DR, and CD38. Women using IPCs had more cervical target cells expressing CD25, which is found on activated and regulatory T cells ($p = 0.0193$, **Figure 4.5A**). However, there was no difference in the proportion of target cells expressing CD25 between IPC users and women in the follicular phase (**Figure 4.5C**), indicating that the higher proportion of CD25+ target cells in IPC users was driven by the underlying higher target cell frequency, and not by any direct effect of progestin-only contraceptives on T cell activation. IPC users also had a non-significant trend

towards more cervical target cells co-expressing HLADR and CD38 (**Figures 4.5B and D**). Once again, there were no differences in the activation state of target cells in the peripheral blood. Thus, IPC users have not only a higher frequency of cervical target cells, but also more activated CD25+ target cells.

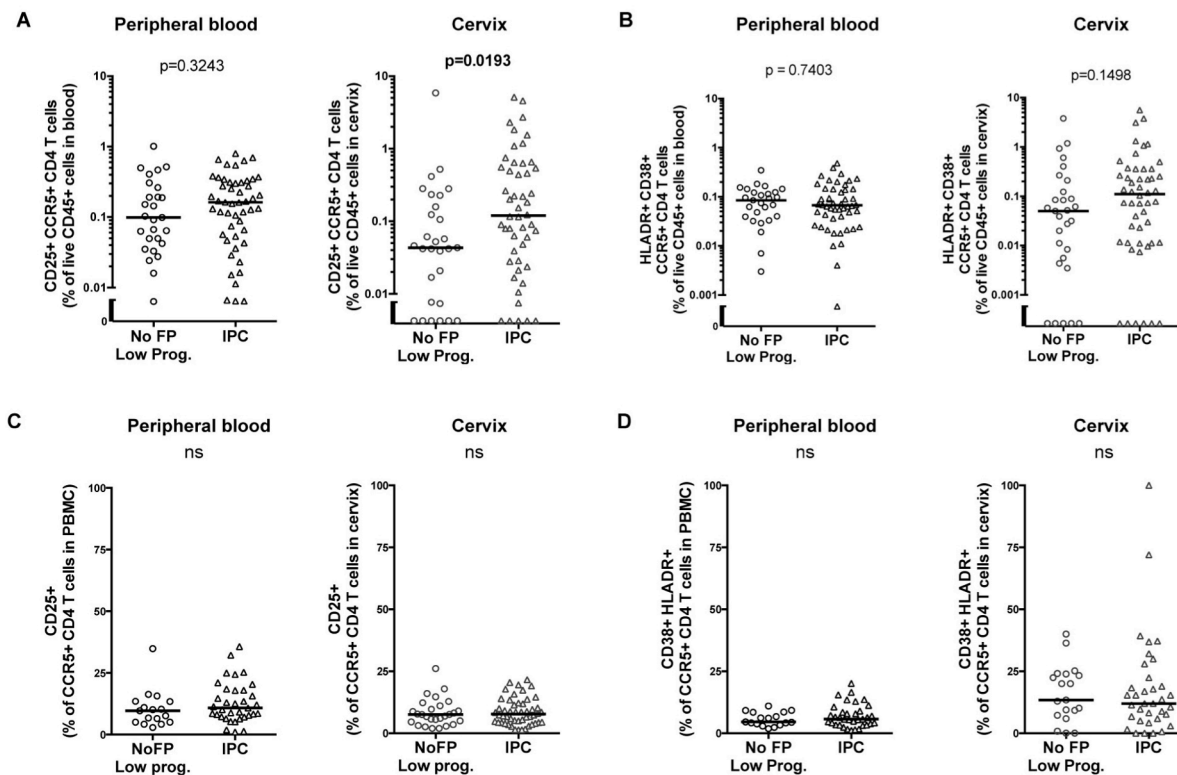


Figure 4.5 IPC use is associated with increased CD25+ activated cervical HIV target cells. Controlling for low endogenous progesterone level, injectable contraceptive use correlates with a higher proportion of activated HIV target cells (CCR5+ CD4+ T cells) amongst all live CD4+5+ cells, with activation defined by (a) CD25 expression, in the cervix but not the blood, and trends toward (b) higher HLA-DR and CD38 co-expression. However, as there is no difference in the proportion of CCR5+ CD4+ T cells expressing (c) CD25+ or (d) HLA-DR+ CD38+. Only cytobrushes with over 500 live CD4+5+ cells were included in the analysis.

Since we found that women with high exogenous progestins in the form of an injectable contraceptive have higher target cell frequencies, we then asked whether endogenous progesterone had the same effect. Only women abstaining from any contraceptive, except for condoms, were considered. We compared women in the follicular phase (endogenous progesterone ≤ 0.3 ng/mL) to those in the luteal phase (endogenous progesterone ≥ 1.2 ng/mL). We found a modest but significantly higher target cell frequency ($p=0.0489$, **Figure 4.6A**) and

more CCR5 expressing CD4⁺ T cells ($p=0.0357$, **Figure 4.6B**) in women in the luteal phase compared to those in the follicular phase. These findings support a role for both endogenous and exogenous progestins in modulating the frequency of cervical target cells.

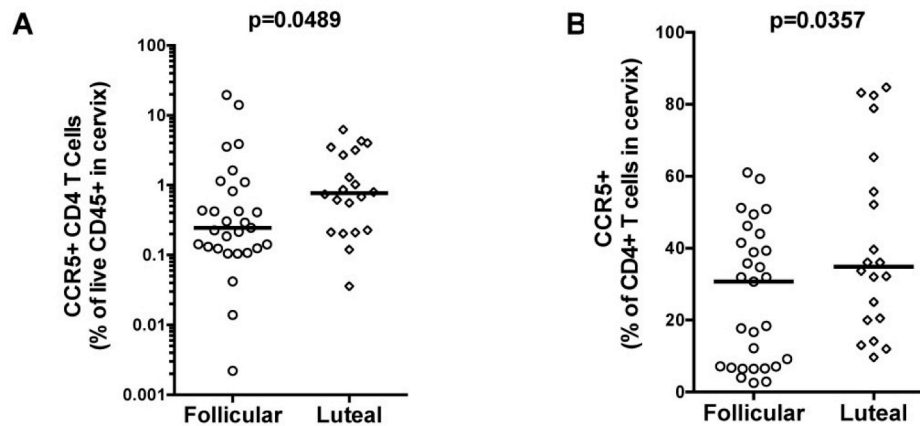


Figure 4.6 (A) Increased target cell frequency in the cervix and (B) increased CCR5 expression on cervical CD4⁺ T cells are associated with a naturally high progestin state. Only women abstaining from any contraceptive, except for condoms, were considered. The follicular phase was defined as an endogenous progesterone level less than 0.3 ng/mL, and the luteal phase was defined as an endogenous progesterone level greater than 1.2 ng/mL. P value refers to Mann-Whitney test. Only cytobrushes with over 500 live CD4⁺ cells were included in the analysis.

Women using injectable progestin-only contraceptives also have simultaneous suppression of both endogenous estradiol production (**Figure 4.2B**). Estradiol has independent effects from progesterone, such as increasing the thickness of the vaginal epithelium (26, 27) and decreasing T cell migration *in vitro* (28, 29). We did not find a difference in target cell availability amongst women who were not using family planning and had low (<0.3 ng/mL) progesterone when we compared women with an estradiol concentration below 50 pg/mL to those with a concentration above 90 pg/mL (**Figure 4.7**), though there was a trend towards lower CCR5 expression with high estradiol. Thus, progestin level, rather than concomitant effects on estradiol level, appears to account for the difference in cervical target cell frequency in a high-progestin state.

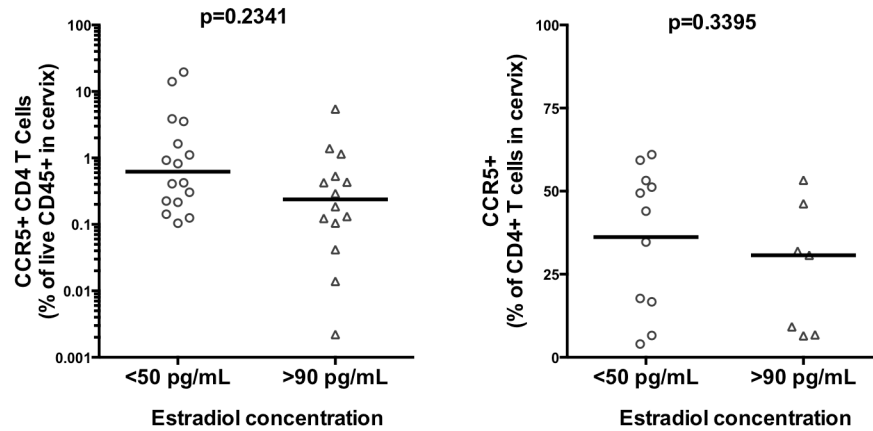


Figure 4.7 Controlling for low progesterone, there is no difference in cervical target cell frequency when comparing women with low vs. high estradiol concentrations, Only women who were not using a family planning method and cytobrushes with over 500 live CD4+5+ cells were included in this analysis.

To understand whether a difference in concentration of soluble cytokines or chemokines could help explain the differences in target cell frequency at the cervix based on progestin state, we compared the levels of 19 cervicovaginal lavage (CVL) cytokines. There was a non-significant trend towards higher CCL5 (RANTES) concentrations in DMPA and Nuristerate users (**Figure 4.8**). IL-10 concentrations were significantly different across groups ($p=0.022$, ANOVA, **Figure 4.8**) before correcting for multiple comparisons. The remaining cytokines showed no trend. Thus, menstrual cycle phase and IPC use do not have distinct genital cytokine signatures in this cohort.

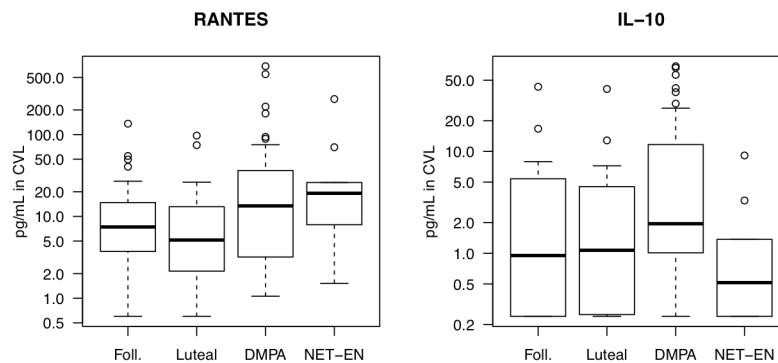


Figure 4.8 Cervicovaginal lavage concentrations of CCL5 (RANTES) and IL-10 among naturally cycling women in the follicular phase ($n=33$) or luteal phase ($n=16$) of the menstrual cycle, or women using DMPA ($n=41$) or NET-EN ($n=10$).

Finally, we asked whether there were any differences in the cervical microbiota between IPC users and women not using family planning. Neither linear discriminant analysis at the species level nor a Fisher's exact test of cervicotype distribution revealed any significant bacterial differences. It is unlikely that bacterial differences are driving the increased target cell frequency in IPC users.

Discussion

In this cohort, as in several studies published previously (6-11, 30), we observed that a strikingly higher proportion of women who used IPCs became infected with HIV than women who did not use a non-condom family planning method. When we examined Nuristerate and DMPA separately, we found that Nuristerate users were at greater risk than those not on family planning, and the risk with Nuristerate was higher than with DMPA. This finding agrees with some (31, 32), but not all previous studies (33, 34). Due to the small number of participants in this sub-IPC analysis, the confidence interval was very large though still significant. Our results support that IPCs generally, not just DMPA, increase HIV acquisition risk.

Behavioral and demographic confounders have traditionally obscured a definitive analysis of an association between IPC use and HIV acquisition risk (35). In the present study, participants were from a narrow age range and one residential area. This necessarily minimized the behavioral and demographic differences between women using IPCs and women not using a family planning method. Surprisingly, we still observed a few behavioral and demographic differences: women using IPCs were older by a year, had sexual partners who were a year older on average, and were having about one more sex episode per week (**Table 4.1**). These differences are consistent with trends of IPC use across South Africa (13).

We used a Cox proportional hazard model, with HIV acquisition as the dependent variable, to assess each potentially confounding variable with IPC use. IPC use remained highly significant even when the age of the sexual partner and sexual frequency were included as

variables. Thus, behavioral and demographic confounders could not explain the profound increase in HIV acquisition risk in this cohort's IPC-using group, so we pursued a biological explanation for this increased risk.

In vitro studies have suggested that endogenous reproductive hormones and exogenous progestins may affect immune cell function (15, 16, 36-38). High progesterone in non-human primates also caused increased SIV transmission (39-41). We therefore assessed the immunological environment of the FGT in the context of IPC use and found that women using IPCs had a significantly higher HIV target cell frequency and expression of CCR5 on cervical CD4+ T cells (**Figure 4.4**). The increased expression of CCR5 on CD4+ T cells in the context of IPC use could be due to increased expression of CCR5 on existing CD4+ T cells, prevention of down-regulation of CCR5, increased trafficking of CD4+CCR5+ T cells to the FGT, increased migration to the cervical epithelial surface where the cells are sampled using the cytobrush, or proliferation of existing CD4+CCR5+ T cells in the FGT. Increased migration of CCR5+ CD4+ T cells to the epithelial surface would be consistent with the study by Mitchell *et al.*, which did not find a difference in total CD3+CCR5+ cell frequency upon immunohistochemical characterization of vaginal biopsies from women on DMPA, but did not assess cellular localization (42). The increased frequency of activated target cells could have important implications for the early stages of HIV infection because activated target cells are not only infectable but also support large amounts of viral replication (23), facilitating viral dissemination from the initial local infection in the FGT (15, 25).

Different types of progestins – DMPA, NET-EN and endogenous progesterone – may each have a unique influence on immune cells, although they may also act through common pathways because of their structural and biological similarity (15, 43). To begin characterizing the mechanism behind increased target cell frequency in the context of IPC use, we further stratified the cohort based on progestin exposure. Women in a high progestin state (naturally cycling and with high endogenous progesterone or using DMPA or NET-EN but with low

endogenous progesterone) all had increased HIV target cell frequency in the cervix compared to women in a low progestin state (naturally cycling with low endogenous progesterone) (**Figure 4.4 and 4.6**). There was no difference among high-progestin states, suggesting that they may have at least overlapping immunomodulatory effects to all be associated with increased target cell frequency at the cervix.

Although the use of IPCs, particularly DMPA, has been of particular interest with respect to increased HIV acquisition risk, the luteal phase of the menstrual cycle has also been described as a time of high risk of HIV acquisition (39, 44, 45). The luteal phase is characterized by high endogenous progesterone. By using both LMP and plasma progesterone level to define follicular and luteal phases, there was a small but significant increase of HIV target cell frequency in the luteal phase, just as in the context of IPC use (**Figure 4.6**).

The level of estradiol fluctuates over the course of the menstrual cycle and is suppressed with IPC use (**Figure 4.2B**), but the cervical immune cell frequency did not differ based on estradiol level (**Figure 4.7**). This suggests that the effect of increased target cell frequency is primarily driven by progestin level, rather than by the low-estrogen state accompanying IPC use or by estradiol fluctuations during the menstrual cycle (11, 46, 47). Furthermore, differences in cytokine concentrations do not appear to drive the difference in HIV target cell frequency, because there was no difference in cytokine concentrations, in contrast to previous work (48).

By analyzing the effect of IPCs on the FGT immune environment *in situ*, we propose a plausible biological mechanism for the significantly increased risk of HIV acquisition in women using IPCs. Although this increased risk has been previously reported epidemiologically, the women using IPCs in the present study have few differences in other HIV risk factors compared to their peers using no hormonal contraceptive. However, latent behavioral and demographic confounders still make any epidemiological assessment of risk difficult to interpret. Our in-depth assessment of the FGT immune environment under the influence of these IPCs provides a

powerful explanation for why women using IPCs acquire HIV at such high rates. Our interrogation of different progestins suggests that increased HIV acquisition risk is likely driven by a high progestin state, as supported by previous studies in model systems (39, 44, 45). This significance of this analysis is two-fold. First, our findings in naturally cycling women, comparing progesterone level and menstrual cycle phase, further support that these differences in cellular phenotype and frequency are not due to behavioral or demographic confounding. Second, if the target cell effect is truly mediated by both exogenous and endogenous progestins, as our data suggest, then increased HIV acquisition risk may impact not only women using IPCs but also naturally cycling women in the luteal phase. In that case, research and policy efforts should shift to understanding the mechanism of increased target cell availability and to developing a biological prophylactic to inhibit that mechanism.

Materials and Methods

Study cohort and sample collection

HIV-negative women ages 18-23 were recruited for the Females Rising through Education, Support, and Health (FRESH) study, a 12-month non-randomized, closed cohort, prospective study conducted outside of Durban, South Africa. Participants were eligible for the study if they were able to understand the information and consent forms, willing to adhere to study requirements, willing to have HIV testing performed twice-weekly, and willing to have samples stored. Participants were excluded from the study if they were pregnant, anemic, or enrolled in any other study.

Twice a week, participants attended classes focused on personal empowerment, job skills training, and HIV prevention, and had a finger prick blood draw for HIV RNA testing. A baseline demographic assessment was administered by a counselor in private upon enrollment. Participants had a pelvic exam, phlebotomy, and completed a counselor-administered HIV risk

questionnaire every three months. All participants received intensive HIV-prevention education and counseling, free condoms, and sexually transmitted infection testing. The study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (Durban, South Africa) and the Massachusetts General Hospital Institutional Review Board (Protocol 2012P001812/MGH; Boston, MA). Informed consent was obtained after the nature and possible consequences of the study were explained.

Procedures

A single nurse performed all of the pelvic exams, during which a posterior fornix swab was used for sexually transmitted infection (STI) testing, a cervicovaginal lavage (CVL) with 5 mL of normal sterile saline was performed using a sterile Pasteur pipette, and cellular collection was performed by fully inserting a cytobrush (Cytobrush Plus GT, Cooper Surgical) into the endocervical canal and rotating twice exactly 360°. Immediately after collection, the cytobrush was placed into antibiotic supplemented RPMI media with 10% FCS. The cytobrush and CVL were stored at 4°C for 1-3 hours during transport to the laboratory. In the laboratory, the CVL was centrifuged at 4°C to fractionate the cellular component from the supernatant; the supernatant was aliquoted and stored at -80°C. Plasma was isolated by centrifuging peripheral blood collected in ACD vacutainers for 15 min at 2600 rpm and aliquoting the plasma supernatant. Peripheral blood mononuclear cells were isolated from the remaining material using a standard Ficoll gradient.

Sexually transmitted infection (STI) detection

STIs were tested independently at Global Labs (Durban, South Africa) from a posterior fornix swab. *Neisseria gonorrhoea* and *Chlamydia trachomatis* were tested using the GeneXpert CT/NG assay (Cepheid). *Trichomonas vaginalis*, HSV-1, and HSV-2 were tested by real-time PCR on a LightCycler (Roche). All positive results for *Trichomonas*, HSV-1, and HSV-2 were

retested, and inconsistent results were sent to an outside lab for a third confirmatory test. Participants with positive STI results were referred for treatment at an outside facility.

Progesterone and estradiol measurement

Plasma progesterone and estradiol levels were measured by the Massachusetts General Hospital Clinical Laboratory Research Core (MGH CLR) using Chemiluminescent Microparticle Immunoassays (CMIA) (Abbott Laboratories). Progesterone values below the limit of detection (0.1ng/mL), values were read as half the minimal limit of detection (i.e. 0.05ng/mL). Estradiol values below the limit of detection (10pg/mL), values were read as half the minimal limit of detection (i.e. 5pg/mL). Progesterone cutoffs for follicular and luteal phases were determined based on the CMIA package insert.

Immunophenotyping of cervical cytobrush samples

Cervical cells were dislodged from the cytobrush and washed. Cell surface staining was performed using monoclonal antibodies to the following human proteins: CD4+5 (HI30), CD3 (UCHT1), CD8+ (SK1), HLA-DR (G46-6), CD38 (HIT2), CD25 (2A3), CCR5 (2D7), CD11c (B-ly6), CD14 (M5E2), CD19 (HIB19) from BD Biosciences; and CD4+ (S3.5) from Life Technologies. All antibodies were titrated before use. Viable cells were identified by a LIVE/DEAD violet stain (Life Technologies). Rainbow beads (Spherotech) were used to ensure comparable fluorescence measurements between experiments. Cells were passed through a 70µm filter prior to analysis on the FACS Aria III (BD Biosciences). Fluorescence minus one controls for CD38, HLA-DR, CD25, and CCR5 were performed on peripheral blood mononuclear cells in each experiment. Cells were sorted directly into TRIzol (Life Technologies), vortexed vigorously, and stored at -80°C. Data were processed using FlowJo software (Treestar). CD4+ T cells were gated by: FSC vs. SSC, singlets, live CD19-, CD4+5+, CD3+, CD4+ CD8-. CD8+ T cells were gated by: FSC vs. SSC, singlets, live CD19-, CD4+5+,

CD3⁺, CD4⁺ CD8⁺ . Antigen presenting cells were gated by: FSC vs. SSC, singlets, live CD19⁻, CD4⁺5⁺, HLA-DR⁺ CD3⁻, and CD11c⁺ or CD14⁺.

Cytokine measurements in CVLs

Cytokines were measured using a human cytokine/chemokine multiplexed bead assay (Millipore) for IL-1 β , IL-2, IL-4, IL-6 IL-8, IL-10, IL-12p70, IFN- γ , TNF- α , IL-1 α , IL-1RA, CXCL10 (IP-10), CCL3 (MIP-1 α), CCL4 (MIP-1 β), CCL5 (RANTES), sCD40L, G-CSF, GM-CSF, FTL-3L, and IFN- α 2. The multiplexed bead assay was performed according to the manufacturer's protocol. A quality control was included with every plate to ensure reproducibility. Cytokine measurements below the limit of detection were assigned a value of half of the minimum detectable concentration for that cytokine.

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Chapter 5: Conclusions and Broader Impacts

Summary and discussion of main findings

By integrating metagenomic, immunologic, transcriptional, and behavioral datasets from a population of young South African women, we demonstrate that endogenous alterations in cervicovaginal bacteria and exogenous injectable progestin-only contraceptives induce distinct forms of genital inflammation *in vivo*.

Unlike most women in the U.S., a minority of South African women in FRESH had *Lactobacillus* dominant genital communities, with a particularly low prevalence of *L. crispatus*. In this small subset of women, *L. crispatus* maintains its immunologically discreet dominance through a variety of mechanisms. By feasting on the glycogen stored in the cervicovaginal epithelium, *L. crispatus* produces copious lactic acid under anaerobic conditions (1) that acidifies the vagina to a pH as low as 2.8 (2). The growth of other organisms, like *Gardnerella vaginalis* and *Candida albicans*, is inhibited at this pH (3, 4). The low pH may even be protective against HIV by neutralizing cell-free HIV virions (5) and reducing the viability of infected cells carrying HIV (6). But in the context of sexual HIV transmission, the alkaline seminal pH buffers the acidic vaginal pH, thereby reducing lactic acid's potential protective effects against HIV. At steady state *L. crispatus* protects its niche in other ways, such as producing bacteriocins and hydrogen peroxide (7), and physically displacing other bacteria by adhering to the epithelial surface (8). Thus, *L. crispatus* is well-adapted to inhabiting the glycogen-rich niche of the human lower genital tract and has numerous mechanisms to protect its niche.

We demonstrate here that the inflammatory state of a *L. crispatus*-dominant community is low. Co-culture of vaginal epithelial cells with *L. crispatus* induces no additional cytokine secretion than from media alone (**Figure 3.3**). In cervicovaginal lavages from women with *L. crispatus* dominance, IL-1 α and IL-1 β concentrations are very low, and TNF- α and IFN- γ are undetectable (**Figure 2.6**). The cervical antigen presenting cells are not activated (**Figure 3.2**).

and the CD4+ T cell frequency is low (**Figure 4.6**). Overall, *L. crispatus* bacterial colonization is associated with a low inflammatory state and likely low HIV acquisition risk.

In contrast, the majority of women in our cohort either had a community composed of *Gardnerella* and *Atopobium vaginae* (CT3) or *Prevotella*, *Shuttleworthia*, *Sneathia*, *Gardnerella*, *Mobiluncus*, and *Megasphaera* (CT4) (**Figure 2.1**). CT4 is a much richer cervicovaginal community, with a higher overall bacterial count (9) that contributes to a scarcity of glycogen for fermentation, causing bacteria to turn to glycerol as an alternative energy source (10). The pH rises above 5.5 (11) from the lack of lactic acid fermenting bacteria and excess tyramine (12). Overall, our shotgun sequencing (**Figure A2.2**) and the work of others demonstrates that polymicrobial CT4 communities produce a dramatic shift in the microbial functions present in the genital tract from that of an *L. crispatus*-dominant community.

We found that CT4 bacteria are associated with high concentrations of IL-1 α , IL-1 β , TNF- α , IFN- γ , IL-10, and IL-8 in the genital tract (**Figure 2.6**). In this thesis, we also elucidated facets of the immunological response underlying this cytokine signal. Vaginal epithelial cells performing their barrier function produce IL-1 α , IL-1 β , and IL-8 in response to CT4 community members such as *Prevotella amnii*, *Mobiluncus mulieris*, *Sneathia sanguinogens* and *S. amnii*, (**Table 3.2**). Disruption of the mucus barrier by sialidases made by bacteria like *Prevotella* and *Gardnerella* (10) may accentuate this response due to closer contact between vaginal epithelial cells and bacteria. The mechanism of epithelial cell IL-1 β secretion may be from sensing bacterial dsRNA through TLR3 or RIG-I, leading to caspase-1 activation and cleavage of pro-IL-1 β into its active form (**Figures 3.4-3.6**). The released active IL-1 β acts on nearby stromal cells, such as endothelial cells and fibroblasts. The endothelial response to IL-1 β includes increased adhesion and recruitment of neutrophils and monocytes (13), and cervical fibroblasts respond by increasing collagen and glycosaminoglycan synthesis (14). Active IL-1 β can also have effects on local immune cells, like antigen presenting cells. The cervical antigen presenting cells that we focused on were macrophages and dendritic cells, due to exclusion of CD19+ B cells in

the FACS sorting panel (**Figure 3.1C**). IL-1 β was predicted to be an upstream regulator of the CT4-associated APC transcriptional signature, and *IL1B* was also one of the genes upregulated in CT4 APCs (**Figure 3.2**).

CT4 APCs also had a profound resemblance to LPS-treated monocytes and dendritic cells when compared to CT1/2 APCs (**Figure 3.2**). This is not surprising since we found that CT4 bacteria produced significantly more LPS than Gram-positive *Lactobacilli* (**Table A2.2**). As cervical APCs reside underneath the epithelium or in the lamina propria (**Figure 3.1A,B**), the exact mechanism by which APCs contact bacterial LPS is unclear. APCs may extend dendritic processes into the lumen, or there may be damage to the epithelial barrier that allows luminal bacteria or their bacterial products to directly access the subepithelial APCs. This damage could come from direct bacterial invasion (15) or from disruption of the tight junctions comprising the epithelial barrier by TNF- α and IFN- γ , which were clearly in the cervicovaginal secretions (**Figure 2.6**) (16, 17).

Regardless of the route of LPS exposure, CT4 APCs appeared activated, with higher CD80, ICAM-1, and MHC class II gene expression levels (**Figure 3.2**). CT4 APCs also upregulated inflammatory cytokine expression such as *IL1A*, *TNF*, and *IL6*, in addition to the aforementioned *IL1B*, and chemokines such as *CCL4*, *CXCL9*, and *CCL8*. Activated dendritic cells within the APC pool may be carrying antigen to the draining lymph node and inducing a T cell response specific to these bacteria. This process may have driven the increased cervical CD4⁺ T cell frequency seen in **Figure 2.8**, but further experiments are needed to demonstrate this connection. Local T cells, as well as local natural killer cells, produce IFN- γ and may have contributed to the higher IFN- γ concentration seen in CT4 CVLs (**Figure 2.6F**). IFN- γ was also predicted to be an upstream regulator of the CT4 APCs.

Finally, the increased CVL IL-10 concentration (**Figure 2.6G**) in conjunction with the APC transcriptional signature (**Figure 3.2A**) suggested that inflammatory monocytes may be attempting to resolve the mucosal inflammation. *ARG1*, *PTGS2*, *IL10*, *IDO1*, *CCL4*, *IL6*, *IL1A*,

and *IL1B* were upregulated both in our CT4 APCs and FACS-sorted mouse intestinal inflammatory monocytes from a *T. gondii* infection mouse model (18). Additional genes upregulated in CT4 APCs, such as *THBS1*, *NEK7*, *OSM*, and *PLEK*, are also involved in wound healing processes. Interestingly, the *T. gondii* mouse model demonstrated that inflammatory monocytes inhibited neutrophil activation and limited tissue damage during pathogen-induced inflammation (18). It has been previously noted that bacterial vaginosis surprisingly does not necessarily have a neutrophilic infiltrate (19) despite elevated IL-8 concentrations (**Figure 2.6H**), and it is tempting to speculate a role for inflammatory monocytes in this observation.

Overall, the characteristics of the complex inflammatory response to CT4 bacteria is becoming increasingly clear, as is the link to HIV risk. We found a trend towards increased HIV acquisition risk among women with CT4 bacterial communities compared to those with *Lactobacillus crispatus* dominance, with a hazard ratio of 3.5. We also found particular bacterial taxa robustly enriched in women who became infected, including several *Prevotella*, *Sneathia*, *Peptoniphilus*, and *L. iners* taxa. This is supported by prior observations that pro-inflammatory cytokines in CVLs are associated with an over 3-fold increased risk of HIV acquisition in women (20) and that HIV-1 exposed women who remain seronegative have lower levels of genital IFN- γ , IL-1 α , and CXCL10 (21).

Of the several possible mechanistic links between CT4 bacteria and HIV acquisition, the increase in activated HIV infectable CD4⁺ T cells in the cervix of women with high pro-inflammatory cytokines provides a potential cellular route. Additionally, damage to the columnar epithelial barrier of the endocervix by cytokine-mediated disruption of tight junctions may enable both bacterial and viral translocation (16, 17). TNF- α and IL-1 β also stimulate HIV replication through NF- κ B activation (22), especially in HIV-1 clade C viral infections that are the most prominent subtype in sub-Saharan Africa (23). Thus, our study identifies several ways in which the predominant cervicovaginal communities found in our cohort of South African women impact genital inflammation and thereby likely modulate HIV acquisition risk.

Independent of cervicovaginal bacteria, we found that women using an IPC were five times more likely to acquire HIV than women not using a family planning method. Among IPC users, women using Nuristerate were infected at higher rates than those using Depo-provera. Though IPC users were more likely to have slightly older sexual partners and have one more sexual act per week, IPC use remained significantly associated with HIV acquisition when these factors were incorporated into a Cox proportional hazards model. In search of a biological mechanism behind this increased risk, we assessed CD4⁺ T cell frequency and phenotype in the cervix and peripheral blood. We found that IPC users on average had a higher frequency of CCR5⁺ CD4⁺ T cells in the cervix due to higher CCR5 expression levels on the cell surface (**Figure 4.4**); this could from an upregulation of CCR5 expression on CD4⁺ T cells, prevention of CCR5 downregulation, or preferential localization of CCR5⁺ CD4⁺ T cells at the epithelial surface that is sampled with a cytobrush. The observed increase of CD25 expression on target cells in IPC users (**Figure 4.5**) may indicate higher cellular activation or an increase in Tregs, which have been shown to be induced by progesterone (24). There was a non-significant trend towards more cervical CD38⁺ HLA-DR⁺ target cells with IPC use, suggesting that activation is at least a component of the CD25 signal. Conversely, we did not see differences in any genital cytokine concentrations in IPC users compared to women not using family planning in the follicular or luteal phase, nor did we see differences in the genital bacterial microbiota (**Figure 4.8** and **A3.1**). While the mechanism behind the increased target cell frequency in cervical tissue of IPC users is still unclear, these data support the hypothesis that IPC use increases HIV acquisition risk. The IPC mechanism is largely distinct from that of diverse bacterial communities, though there may be common path with increased target cell frequency.

For the first time in humans, we were also able to assess the dynamics of HIV dissemination in acute infection. We found that the peak CVL viral load was about a week before the peak plasma viral load (**Figure 2.10C**) and the viral load was detectable earlier in the CVL than the plasma, supporting the hypothesis that the virus first replicates within the genital

mucosal tissue before spreading systemically. Although anecdotal, two women with *L. crispatus* and *L. iners* vaginal bacterial communities had nearly undetectable CVL viral loads throughout acute infection despite having millions of viral copies per mL of plasma (**Figure 2.10B**). These two women also had low levels of pro-inflammatory cytokine levels within the bacterial signature, suggesting that HIV does not replicate effectively in the genital tract when inflammation is not present. We also determined a viral cytokine signature that was generally distinct from the CT4 bacterial cytokine signature that we had defined previously. Genital levels of CXCL10, CCL5, sCD40L, GM-CSF, CXCL8, and TNF- α were significantly higher at peak viral load than before infection.

Finally, because the gut bacteriome is significantly altered in chronic HIV infection (25-27), we thought that the vaginal bacteriome would also shift in acute HIV infection when the genital viral load reached 10,000 to 1,000,000 copies per mL and there was a massive depletion of CD4⁺ T cells. Though our sample size of 15 was small, we did not see significant changes in bacterial species abundance between acute and chronic infection (**Figure 2.12**), nor in overall community stability compared to HIV negative women (**Figure 2.14**). The only phylum that was significantly different in chronic versus acute infection was an unknown *Rickettsiales* family member, which was present at greater than 10% abundance in several women at the first week post-infection but decreased in abundance at chronic infection (**Figure 2.13**). We also saw a significant reduction in HIV-positive women with CT1 bacterial communities compared to HIV-negative women (**Figure 2.14**), as previously reported (28), but we believe this is likely a consequence of fewer women with CT1 communities becoming infected in the first place. Overall, the surprisingly stability of cervicovaginal bacterial communities in HIV infection suggests that these bacteria do not rely on CD4⁺ T cells for survival and are not significantly affected by changes in the cytokine milieu.

Strengths and weaknesses of approaches taken

Focusing on the FRESH cohort of young, sexually active women

Strengths: The FRESH study provided a unique opportunity to focus on understanding the causes of genital inflammation in a demographic that is particularly vulnerable to HIV infection: 18 to 23 year-old women in an area with high HIV prevalence. This is the target demographic for novel pre-exposure prophylaxis methods that are female controlled and HIV vaccines that are in development. Since microbicides like intravaginal tenofovir gel have been shown to fail in women with high inflammation, we hope that understanding the causes of inflammation in the same group that will be using these interventions will inform and improve their efficacy.

FRESH also offered several logistical advantages. At the start of the study we established the mucosal sampling methods to collect the appropriate specimens to answer our scientific questions in a sterile and reliable manner. We trained the dedicated nurse who performed all the pelvic exams herself, ensuring consistency across samples. We also trained the lab staff at the Harvard Pathogenesis Program at the University of KwaZulu-Atal School of Medicine to preserve cell viability and maximize yields when isolating cells from the cervical cytobrush and peripheral blood samples.

We performed the experiments described in this thesis while the study was on-going, which enabled us to perform on-going quality control checks and perform experiments on fresh samples. For example, we were able to perform cellular phenotyping and sorting at the KwaZulu-Natal Research Institute for Tuberculosis and HIV (K-RITH) within hours of sample collection, thereby avoiding a freeze-thaw cycle and maintaining viability of the fragile cervical cells. Unlike studies that utilize archived samples from completed clinical trials, we were able to perform cervicovaginal lavage cytokine measurements within approximately six months of their

collection, avoiding degradation of cytokines during storage. Thus, we are very confident in the quality of the samples that we utilized.

The participants' twice-weekly visits to the study site over the course of a year also afforded numerous advantages. The participants received intensive counseling about sexual health, HIV, and the goals of the mucosal sampling, and received the benefit of unprecedented access to medical care and three-monthly sexually transmitted infection testing. As a result of this education and the concurrent classes focusing on empowerment and employment, the participants were very invested in the study. We believe this translated to honesty in the behavioral questionnaires, as we could validate their self-reports of contraceptive usage by measuring plasma drug levels, last menstrual period with plasma progesterone and estradiol measurements, and last reported sexual activity by measuring prostate specific antigen in CVLs. The high-frequency HIV viral load testing afforded a unique opportunity to identify women within days of HIV infection. The principal investigators were also able to obtain IRB approval to begin very early anti-retroviral treatment, which is on-going.

Weaknesses: One weakness of the study was the lack of involvement of the male sexual partners. It would have been very informative to collect information about the sexual partners' penile microbiome and genital inflammation level. Additionally, our participants were counseled to seek antibiotic treatment for themselves upon receiving a positive STI test results and they were told to disclose to their sexual partners, but we do not know how often this happened. As a result, our participants may have been re-infected with STIs from their untreated partners.

Another weakness was the small size of the study, with an original enrollment goal of 300 women. The intensive nature of the FRESH educational curriculum limited its size.

Alternative approaches: We could have performed higher frequency mucosal sampling with biopsies in women enrolled from Boston, MA. However, in hindsight we were unlikely to have seen the inflammatory signal of CT4 bacteria as the proportion of women in U.S. with this community type is small. We could have also utilized archived samples from a larger, completed

South African clinical study, but we would have had to rely on their pre-determined mucosal sampling conditions and behavioral questionnaires. We would also not have been able to perform meaningful cellular analyses.

Characterizing inflammatory properties of bacteria in women

Strengths: Determining associations between genital bacteria and pro-inflammatory cytokines in women ensured the biological relevance of our findings. It allowed us to determine and focus on the bacterial species that are prevalent in the target demographic for preventative HIV interventions.

Weaknesses: In our observational study in humans, we cannot demonstrate the causal link between particular genital bacterial communities and HIV acquisition. We are limited to showing strong associations in both cross-sectional and longitudinal analyses that argue for causation. Additionally, mechanistic studies are very difficult in humans, without the unprecedented control over experimental conditions afforded by mouse models.

Alternative approaches: NOD *scid* gamma (NSG) mice reconstituted with a human immune system are the only the model system for HIV infection, and non-human primates infected with simian immunodeficiency virus (SIV) or chimeric HIV-SIV viruses (SHIV) offer another very relevant model. The vaginal microbiome of non-human primates vary by species, but are generally not *Lactobacillus* dominant and tend to contain some CT4 bacteria like *Prevotella* and *Sneathia* (29). Both conventional and gnotobiotic mice have proven to be useful in dissecting microbe-host interactions in the gut and skin (30-34). Mouse models for the vaginal microbiome currently do not exist, with the exception of a monocolonization model with *Gardnerella vaginalis* (35). Only one published study has determined the natural vaginal bacteriome of mice and it is more similar to human skin, with colonization mostly by *Acinetobacter* or *Streptococcus* (36). We are currently exploring the possibility of using the humanized mouse model, as discussed in the next section on “Future Studies”.

Bacterial characterization using 16S V4 sequencing and shotgun sequencing from cervical swabs

Strengths: The 16S *rRNA* gene contains nine hypervariable (V) regions, and several primer sets have been designed to amplify different sets of V regions. There is no consensus regarding the optimal V region to sequence for vaginal microbiome studies. While the Human Microbiome Project recommends using the 27F and 338R primer set to amplify the V1-V2 region, this primer set fails to amplify *Bifidobacteriaceae* in general and completely underestimates the abundance of *G. vaginalis* (37). Rob Knight's group developed a robust set of V4 primers using 515F and Golay-barcoded 806R that have been widely used in a variety of settings through the Earth Microbiome Project (38). We decided to use these V4 primers for our study and they effectively amplified *Gardnerella* and enabled us to multiplex over 100 samples on a single MiSeq run.

Weaknesses: Several species within the *Lactobacillus* genera do not have much unique variation in the V4 region. Thus, we had to use the supplementary approaches of shotgun sequencing and oligotyping to distinguish *L. crispatus* from other *Lactobacilli*.

Alternative approaches: David Fredricks' group has investigated the vaginal microbiome using 338F and 806R (39). This primer pair seems to both amplify *Gardnerella* and distinguish between most *Lactobacillus* species.

Pro-inflammatory cytokine measurements from cervicovaginal lavages (CVLs) using a multiplexed bead assay

Strengths: We collected cervicovaginal secretions using a 5 mL saline lavage. After pelleting cellular debris, approximately 4 mL of lavage supernatant remained for future assays. Using regular and high-sensitivity Luminex multiplexed bead assay kits, we were able to measure 19 cytokines in duplicate using only 200 µL of CVL, allowing us to conserve precious material. Luminex kits include internal quality controls and have low inter-plate variability, allowing direct comparisons of samples collected throughout the duration of a study and

assayed on different days. Luminex kits have also been used by many other groups studying CVL cytokine concentrations, including our collaborators at CAPRISA (40), which simplifies inter-study comparisons.

Weaknesses: CVLs necessarily dilute cervicovaginal secretions by 5 to 10-fold. While every effort is made to ensure consistency across sampling, there is inevitably some variation that adds noise to the cytokine measurements. When we compared CVL cytokine measurements collected from the same participant over time, we were pleased to see that intra-individual variability was far less than the inter-individual variability. Both the CVL dilution factor and Luminex assay sensitivity limit contributed to several pro-inflammatory cytokine measurements falling below the minimum detection concentration in CT1 and CT2 women. A more sensitive assay would likely have accentuated the cytokine differences between CT1/2 and CT3/4.

Alternative approaches: Other groups have collected cervicovaginal secretions using an absorbent swab or Weck-Cel sponge, and cervical secretions using a SoftCup diaphragm that fits over the cervix. While these methods collect secretions without dilution, they suffer from difficulty with retrieving cytokines from the swab, sponge, or mucus-rich secretion, and have lower overall protein recovery levels than CVLs (41). An alternative to Luminex would be to use a biomarker platform based on electrochemiluminescent technology (e.g. Meso Scale Discovery, MSD), which has a lower limit of detection that is 5-10 times lower than Luminex's for most cytokines, and also has a lower coefficient of variance. Unfortunately, it also costs about three times more than Luminex and the MSD instruments are not widely available.

Cellular phenotyping from cervical cytobrushes

Strengths: Cervical cytobrushes are a clinically approved and relatively non-invasive form of cellular collection because of their use in Pap smears. Cytobrushes do not require

anesthetics and do not cause bleeding or trauma. The brush primarily samples the superficial surface of the endocervical canal.

Weaknesses: There is wide variation in the number of cells collected using a cytobrush. Some of this variation arises from the mucus contained within the cervical canal, which can adhere to the brush and make it difficult to retrieve single cells. Also, the location of the cervical transformation zone varies amongst women, and whether it is sampled by the cytobrush can affect cell retrieval, as the transformation zone contains a high concentration of leukocytes (42). Another weakness is that the cervical cytobrush does not provide information about vaginal immune cells.

Alternative approaches: In this study, we could have also collected a vaginal cytobrush, which is far less common and would presumably mostly contain superficial vaginal epithelial cells. Ectocervical and vaginal biopsies provide a high cellular yield and we commonly use them in our Boston-based studies, but it would not be ethical to perform biopsies in the FRESH participants due to concerns of increased acquisition risk from epithelial disruption and post-biopsy inflammation.

Validation of pro-inflammatory properties of individual bacterial species using a vaginal epithelial cell co-culture model

Strengths: This reduced system with immortalized human vaginal epithelial cells and single bacterial species enables one to quickly and reproducibly screen for pro-inflammatory cytokine secretion (43). Epithelial cells comprise the majority of the cervicovaginal mucosal surface and directly contact bacteria, making their bacterial responses particularly relevant. Additionally, epithelial cells are likely a major contributor to the cervicovaginal secretions collected with a CVL.

Weaknesses: We tested vaginal bacterial isolates from the U.S. and Sweden, assuming they would be representative of the strains we found in South Africa. We have since started collecting additional cervicovaginal swabs from the FRESH study in a manner that preserves

bacterial viability for future bacterial cultivation. This *in vitro* system is also devoid of mucus, which may spatially separate bacteria from epithelial cells *in vivo*.

Alternative approaches: An alternative to single epithelial cell layers are commercially available 3-D VEC-100 multi-lamellar cultures of vaginal-ectocervical cells, or VLC-100 tissues which also contain dendritic cells. A group at Baylor University has directly placed freshly-collected vaginal swabs into PBS and applied freeze-thawed bacteria from those swabs to the apical surface of VEC-100 tissue to measure cytokine secretion (44). In their paper, they say they preserved the original distribution of bacterial species after freeze-thaw, but this claim is based on PCR from bacterial DNA, which can come from both viable and dead bacteria. Using this method, it is unclear whether host factors from the original swabs, such as pro-inflammatory cytokines, are also preserved and transferred to the VEC-100 tissue. It is worth investigating this approach in future studies.

RNASeq analysis of sorted cytobrush and peripheral blood samples

Strengths: Despite numerous technical and logistical challenges, we were able to perform paired cytobrush cell and PBMC sorting directly into TriZOL, followed by RNA extraction and digital RNAseq using the SCRBS-Seq method. We optimized the phenol-chloroform-IAA (PCI) RNA extraction method to successfully isolate RNA from as few as 20 cells sorted into TriZOL. Unlike most column-based RNA extraction methods, PCI extraction preserved microRNAs and long non-coding RNAs. SCRBS-seq also enabled us to quickly and relatively inexpensively obtain digital gene expression from low RNA inputs.

Weaknesses: PCI-based RNA extraction is extremely laborious and prone to human error, especially when extracting from small cell numbers. In addition, SCRBS-Seq provides gene counts but not isoform information, and the overall read depth using SCRBS-Seq was less than we anticipated, with approximately 100,000 reads per sample. This may have hindered our ability to detect genes with low expression levels in the APCs. While we also performed SCRBS-

Seq on CCR5+ CD4+ T cells, CCR5- CD4+ T cells, and CD8+ T cells in addition to APCs, we were not able to perform deep enough sequencing to extract meaningful information.

Alternative approaches: The SMART-Seq2 method has now been developed to perform full-length transcriptome sequencing from single cells (45) and bulk populations.

Probing the mechanism of IL-1 β production from epithelial cells using human immortalized cell lines

Strengths: Since caspases are activated during apoptotic cell death and pro-IL-1 β can be released from dying cells, it is critical to perform these experiments in a setting with good cell viability. Due to the fragility of epithelial cells *ex vivo*, cervical tissue explants are not a viable model due to variable cell death. However, immortalized human epithelial cells are robust and retain many features of primary epithelial cells. Additionally, the caspase-1 FLICA probe combined with propidium iodide allows one to easily exclude necrotic cells by flow cytometry.

Weaknesses: IL-1 β ELISAs suffer from the problem of not discriminating between pro-IL-1 β and active IL-1 β . Additionally, in the study described in this thesis, we focused on a model of Toll-like receptor agonist priming to upregulate NLRP3 followed by activation with ATP, but there are a variety of inflammasomes aside from NLRP3 that are triggered by danger signals other than ATP.

Alternative approaches: Western blots allow one to distinguish between 31 kDa pro-IL-1 β and 17 kDa active IL-1 β . Utilizing a caspase-1 inhibitor like VX-765 in conjunction with poly(I:C) and ATP stimulation would have more convincingly shown that inflammasome activation specifically was important for IL-1 β release. We could have also used a joint TLR-RLR inhibitor called BX-795 to demonstrate the necessity of poly(I:C) signaling through either of those receptors. Finally, we could have also use siRNAs to knock-down TLR3, RIG-I, and NLRP3 to determine which receptor was most critical for caspase-1 activation and IL-1 β secretion in response to poly(I:C) (46, 47).

***In vivo* approach to determining the influence of IPCs on HIV target cells**

Strengths: Examining the effects of IPCs in women obviously increases the relevance of the findings. This is the first study to our knowledge to demonstrate both increased HIV acquisition risk and cervical target cell frequency with IPC use. Using a mouse animal model would suffer from many limitations due to differences in natural hormone cycles (mice have a 4-5 day menstrual cycle) and anatomic structures (mice have a bicornuate uterus). Some non-human primates, like pigtail macaques, have 30-day menstrual cycles, but despite that similarity there are clearly differences in physiologic responses, such as the profound vaginal thinning effect high-dose progesterone observed in macaques (48, 49) but not humans (50).

Weaknesses: There is high biological variation in cervical target cell frequency in women, due to differences in time since last IPC injection, timing in the hormonal cycle, active and latent STIs, cervicovaginal bacteria, and the presence of cervical ectopy, among other reasons. Thus, seeing a signal amongst this variation requires large sample sizes. Also, as previously discussed, cervical cytobrushes only capture information regarding the cervical immune environment, and it would have been informative to acquire data on vaginal effects.

Alternative approaches: Miller *et al.* performed a well-designed study in Seattle, Washington that enrolled women who wished to begin using DMPA (51). The participants received a vaginal biopsy before receiving DMPA and at every subsequent 3-monthly visit when they came for their DMPA injection. Mitchell *et al.* assessed T cell frequency in the vaginal biopsies collected from that study using immunofluorescent staining (50). While it was convenient for the participants to get their DMPA dose at the same visit as the vaginal biopsy, it may have abrogated their signal because DMPA reaches peak levels about a week after injection, and plasma levels progressively decline thereafter (52). An improvement on this study would be to perform the vaginal biopsies about a month after each DMPA dose.

Future studies

Analyzing cellular transcriptional networks

We plan to extend the antigen presenting cell transcriptional analysis presented in this thesis to also include sorted cytobrush cell populations of epithelial cells, neutrophils, CD4+ T cells, and CD8+ T cells. We anticipate that a transcriptional network analysis of both innate and adaptive immune cells collected at the same time from the same person whose bacterial microbiome has already been determined will provide an *in vivo* snapshot of the genital immune response to bacteria. We have already sorted these cell populations and will use the SMART-Seq2 RNAseq method to achieve a higher read depth (at least 5 million reads per sample) than what we previously acquired with SCRB-Seq. We will also need to develop new analysis tools to determine gene expression changes amongst different cell types. We hope that this comprehensive characterization will also provide new insights into mucosal immunology.

Developing a mouse model to study host-bacterial interactions in the FGT

The utility of model organisms for understanding host-microbiota interactions cannot be understated (reviewed in (53)). While animal models have proven very useful for studying sexually transmitted infections like HSV-2 (54) and chlamydia (55), a robust small animal model to study host-bacterial interactions in the genital tract does not yet exist. We believe the results described in this thesis will provide a metric for evaluating potential models of investigating the role of genital microbiota in genital inflammation. Specifically, a valuable model would show a minimal inflammatory response to *Lactobacillus crispatus* and a pro-inflammatory response consisting of at least IL-1 α , IL-1 β , and TNF- α secretion in response to *Prevotella spp.* Aside from the bicornuate uterus, the mouse lower reproductive tract is anatomically similar to the human's, with a vagina covered by a stratified squamous epithelium, posterior fornix, and single cervix that contains a transformation zone (**Figure 5.1**), making the mouse an attractive potential model.

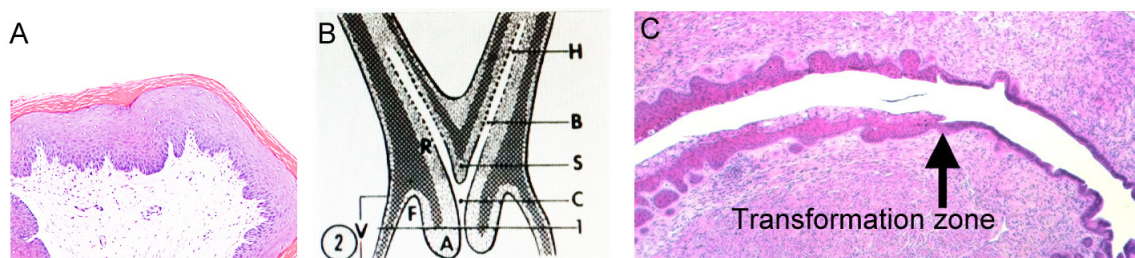


Figure 5.1. Anatomy and histology of the mouse lower reproductive tract. (A) The stratified squamous epithelium of the mouse vaginal wall in estrus. Adapted from the Atlas of Laboratory Mouse Histology (56). (B) Schematic of the mouse cervix (C), also showing vagina (V) and two uterine horns (H). Adapted from Leppi *et al.* (57). (C) The mouse transformation zone, with an arrow pointing to the transition between the squamous epithelium on the left and columnar epithelium on the right. Adapted from Brake *et al.* (58).

We performed a proof-of-concept experiment with Dr. Raina Fichorova using gnotobiotic mice and found that seven days after intravaginal inoculation, mice treated with *P. bivia* had higher vaginal IL-1 β and TNF- α concentrations as well as a vaginal CD4 $^{+}$ T cell infiltrate compared to mice treated with *L. crispatus*, which had comparable pro-inflammatory cytokine levels and CD4 $^{+}$ T cell frequencies to the PBS treated controls. It will be important to determine that CT4 bacterial species like *Prevotella spp.* are not invading the mucosa of these mice, which could explain the vigorous inflammatory response, and whether conventional specific-pathogen-free (SPF) mice have similar responses. Using SPF mice would provide far more opportunities to explore the mechanism behind the inflammatory response, due to the wide variety of mutants available. Alternatively, establishing the model in NOD *scid* gamma (NSG) mice reconstituted with a human immune system via a bone marrow, fetal liver, and fetal thymus transplant would provide an opportunity to challenge the mice with HIV after intravaginal bacterial inoculation (59).

Filling in the metagenomic gaps by examining the virome and mycome

The microbial foci of this thesis were bacteria and HIV, but the virome and mycome are increasingly being appreciated as important contributors to human health (60, 61). The human virome is primarily comprised of four types of viruses: viruses that infect human cells, viruses

that infect parasites that live within us, bacteriophages that infect bacteria that live within us, and genetic elements from viruses that have integrated into the human genome, such as prophages and endogenous retroviruses (60). In collaboration with Dr. Skip Virgin's lab at Washington University in St. Louis, we are characterizing the human vaginal virome and fungome in the FRESH cohort, determining their contribution to genital inflammation, and examining their potential bacterial modulatory effects. From our preliminary data and the work of others (62, 63), we expect the vaginal mycome to contain at least *Candida albicans*, we expect the vaginal virome to contain at least bacteriophages, herpesviridae, anelloviridae, and *Trichomonas vaginalis* virus. Bacteriophages may play a particularly important role in our cohort, as shifts in bacterial communities may actually be driven by bacteriophage changes (64). It is tempting to speculate that bacteriophages induce the vaginal microbial changes that we saw in our longitudinal analyses, by a mechanism similar to Pavlova *et al.*'s discovery of vaginal bacteriophages that preferentially lyse *Lactobacilli* (65).

Extending the observations of IPC effects from humans

Finally, we propose to use a human cervical explant model to directly test the relative effects of progesterone, medroxyprogesterone acetate, norethisterone, and estrogen. We have a protocol in place to receive excess surgical tissue from Massachusetts General Hospital within hours of collection with associated clinical metadata such as the patient's age and prior hormonal therapy. Blocks of cervical tissue oriented with the apical side facing up and edges sealed with agarose can be cultured for at least week (66) with hormones, and then either challenged with HIV, digested and phenotyped by flow cytometry to assess CCR5 expression levels, homogenized to assess RNA expression levels, or formalin fixed and analyzed by immunohistochemistry to assess T cell localization. While cervical explants have their caveats (67), we believe this approach will enable us to validate our *in vivo* findings.

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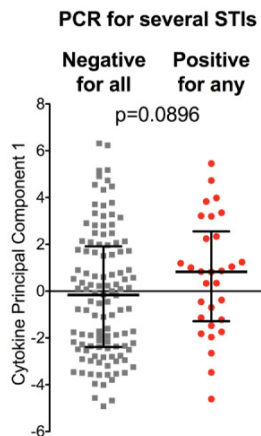
Appendix 1

Corresponding to Chapter 2

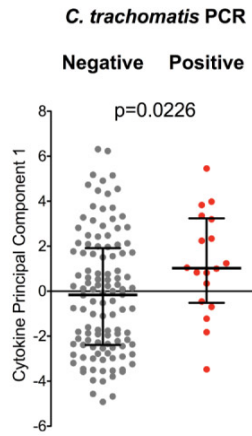
A

	IL-1RA	IP-10	RANTES	MIP-1α	IL-12p70	IL-10	TNF-α	IL-1β	IFN-γ	IL-2	IL-4	FLT-3L	IL-1α	MIP-1β	IFNα2	sCD40L
IL-1RA	1	0.37										0.32	0.31			
IP-10	0.37	1	0.53									0.34		0.37		
RANTES		0.53	1	0.42		0.4	0.32	0.31		0.38	0.31	0.34		0.58	0.38	0.48
MIP-1α			0.42	1	0.33	0.54	0.46	0.47	0.37	0.33	0.34			0.53		0.46
IL-12p70				0.33	1	0.61	0.72	0.5	0.53	0.6	0.42	0.32	0.4	0.38	0.31	0.47
IL-10			0.4	0.54	0.61	1	0.78	0.56	0.48	0.54	0.61			0.44		0.44
TNF-α			0.32	0.46	0.72	0.78	1	0.69	0.55	0.52	0.49		0.46	0.41	0.31	0.36
IL-1β			0.31	0.47	0.5	0.56	0.69	1	0.66	0.49	0.59	0.42	0.61	0.39		0.33
IFN-γ				0.37	0.53	0.48	0.55	0.66	1	0.54	0.63	0.46	0.58	0.39	0.31	0.36
IL-2			0.38	0.33	0.6	0.54	0.52	0.49	0.54	1	0.65	0.45	0.35	0.44	0.45	0.57
IL-4			0.31	0.34	0.42	0.61	0.49	0.59	0.63	0.65	1	0.44	0.43	0.34	0.28	0.47
FLT-3L	0.32	0.34	0.34		0.32			0.42	0.46	0.45	0.44	1	0.6	0.43	0.44	0.52
IL-1α	0.31				0.4		0.46	0.61	0.58	0.35	0.43	0.6	1	0.31	0.31	
MIP-1β		0.37	0.58	0.53	0.38	0.44	0.41	0.39	0.39	0.44	0.34	0.43	0.31	1	0.61	0.6
IFNα2			0.38		0.31		0.31		0.31	0.45	0.28	0.44	0.31	0.61	1	0.67
sCD40L			0.48	0.46	0.47	0.44	0.36	0.33	0.36	0.57	0.47	0.52		0.6	0.67	1

B



C



D

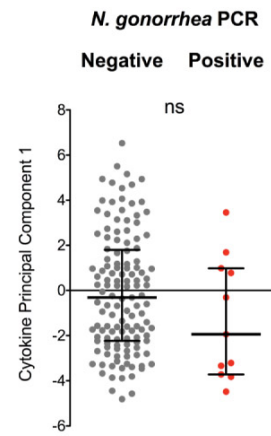


Figure A1.1. Genital cytokine correlations. **(A)** Correlations between cervicovaginal lavage (CVL) cytokine concentrations. Spearman's rho correlation coefficients between CVL cytokines (using log10 normalized concentrations). Only correlations with $p < 0.001$ are displayed. **(B-D)** Genital cytokine levels in women with or without at least one positive STI PCR result (*C. trachomatis*, *N. gonorrhoea*, *T. vaginalis*, HSV-1, or HSV-2) **(B)**, or positive only for *C. trachomatis* versus negative for all STIs **(C)**, or positive only for *N. gonorrhoea* versus negative for all STIs **(D)**. Data shown as median with interquartile range. P values determined by a two-tailed Mann-Whitney test, $n=146$.

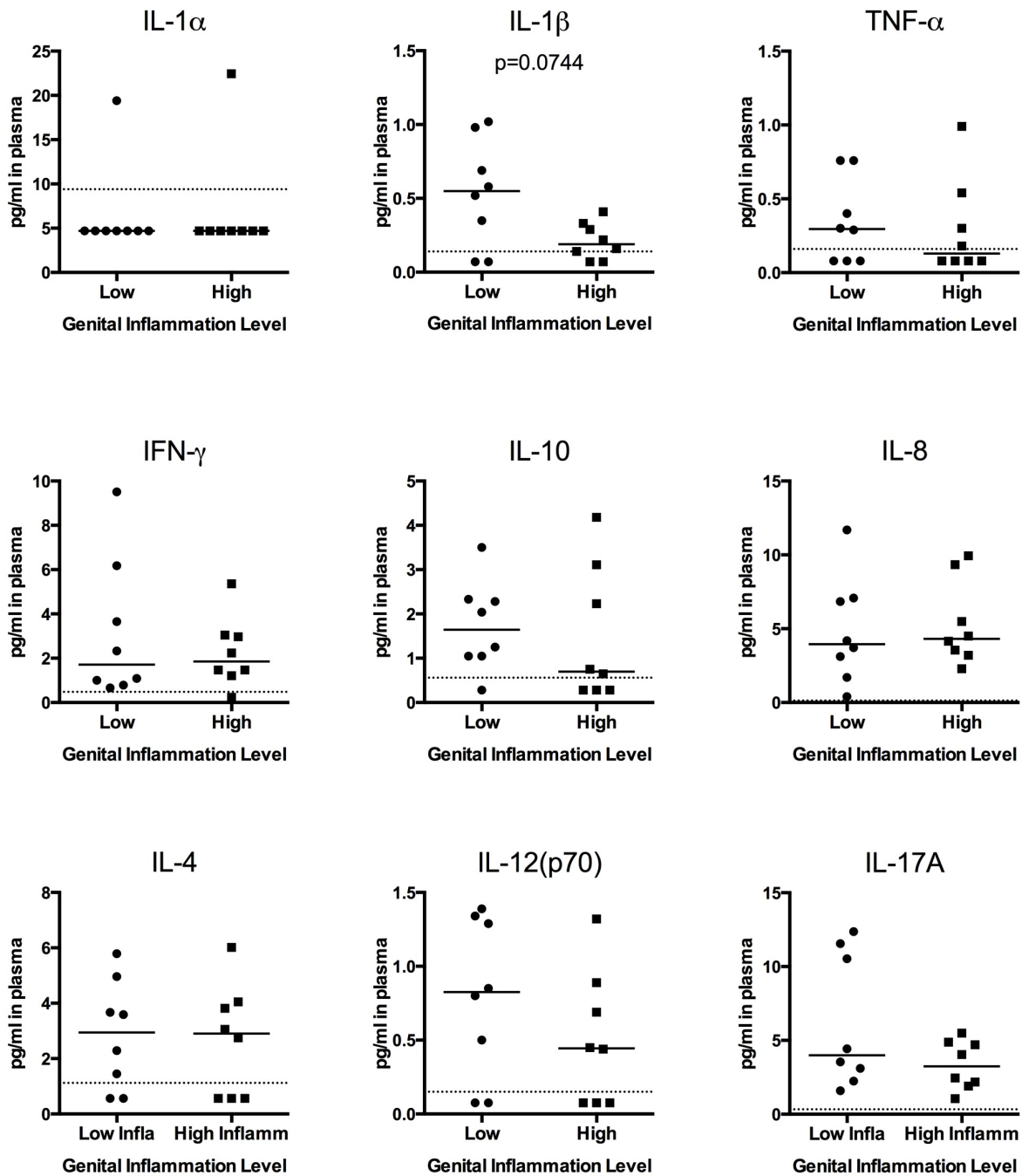
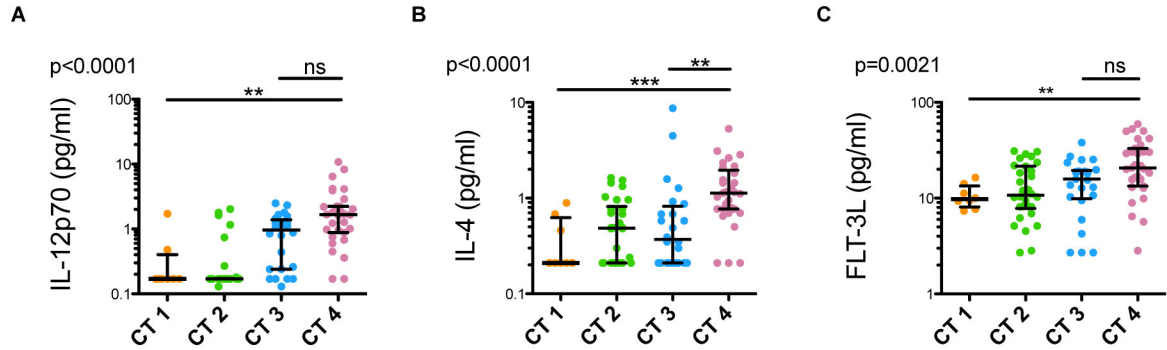


Figure A1.2 Plasma cytokine levels amongst women with the highest or lowest levels of genital pro-inflammatory cytokines (n=16). Dashed lines indicate minimum detectable concentrations. All comparisons are $p>0.3$ by the Mann-Whitney test except for IL-1 β .

Including women with sexually transmitted infections



Excluding women with any sexually transmitted infections:

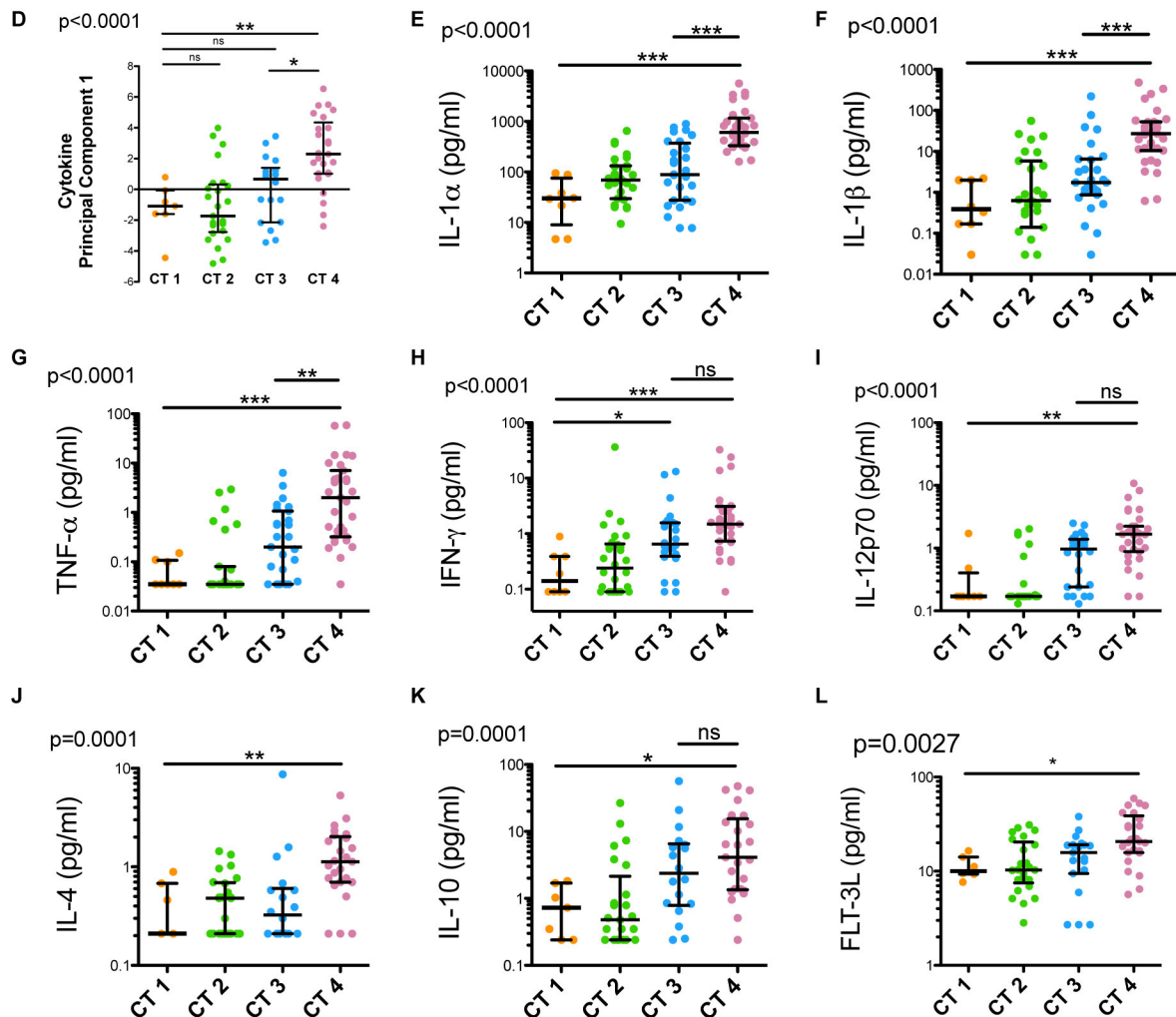


Figure A1.3. Additional genital cytokines that are higher in CT4 than CT1, including women with STIs (A-C) or excluding women with any STIs (D-L). P values in upper left corners were determined by a Kruskal-Wallis test, and asterisks denote significance level after post-test. Data shown as median with interquartile range. * denotes $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

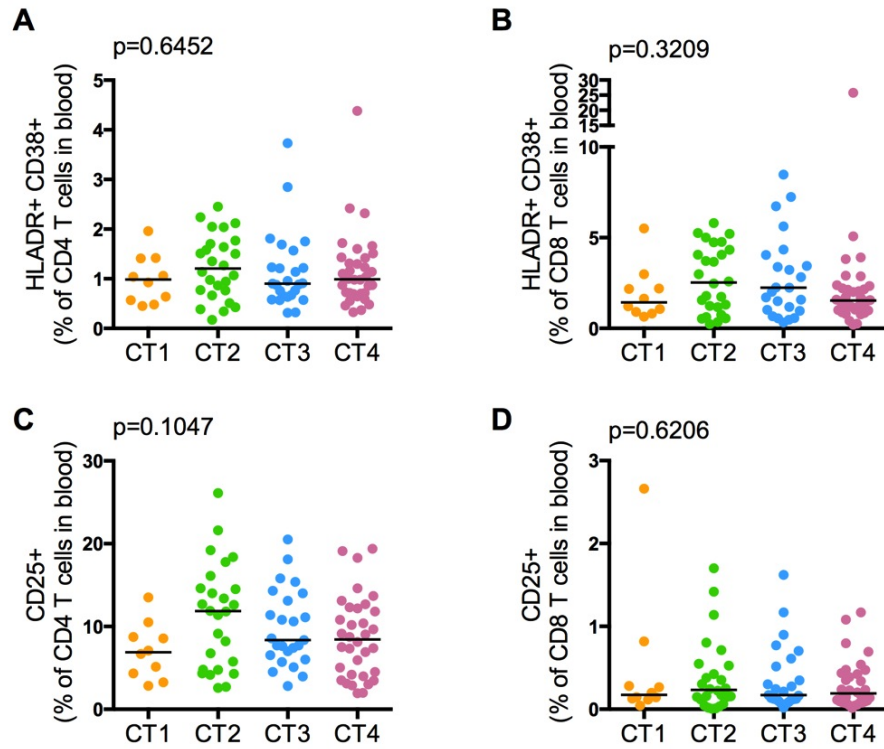


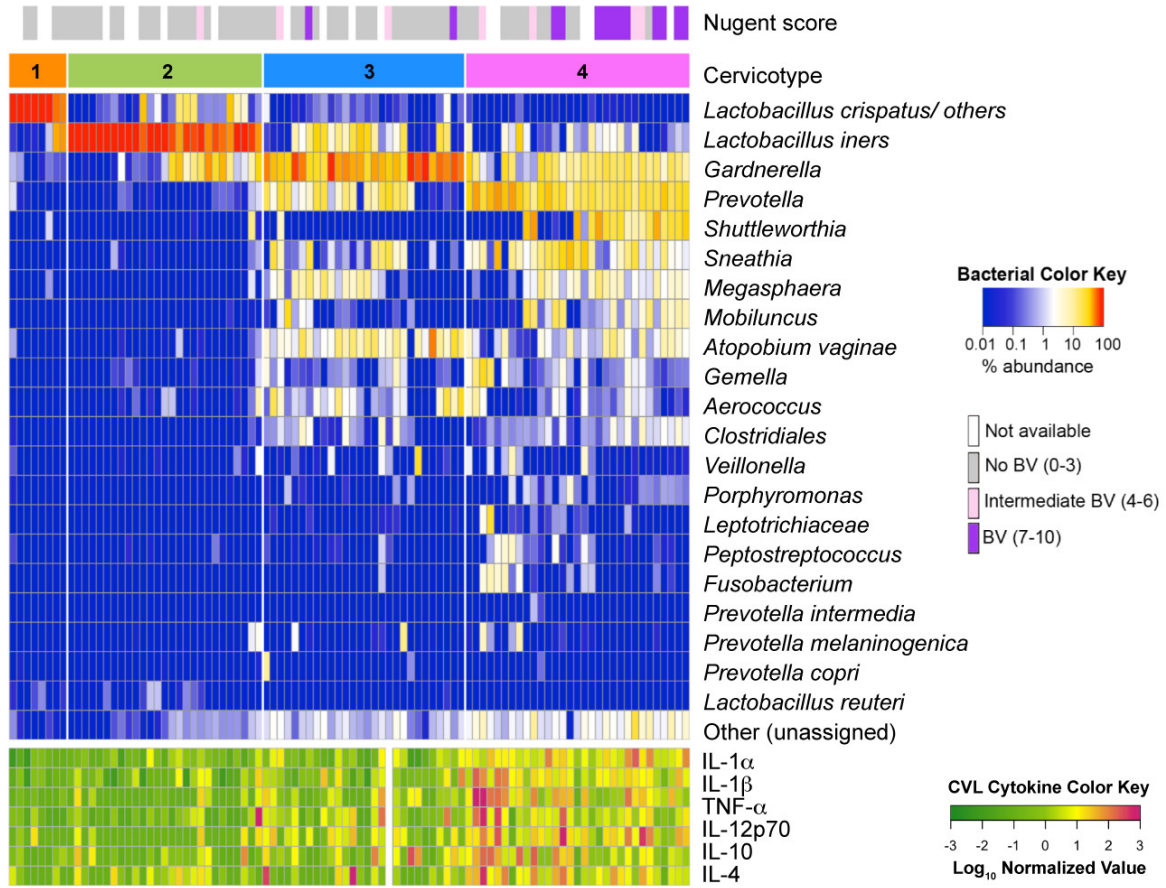
Figure A1.4. Genital cervicotypes do not correlate with peripheral CD4+ (A, C) and CD8+ T cell (B, D) activation levels. Significance levels were determined by a Kruskal-Wallis test for HLA-DR/CD38 and an ordinary one-way ANOVA for CD25.

Figure A1.6 Correlations of bacterial communities with genital cytokines, and bacterial species with one another.

(A) Heatmap of bacterial taxa identified by 16S V4 sequencing with a second heatmap representing matched CVL cytokines: IL-1 α , IL-1 β , TNF- α , IL-12p70, IL-10, and IL-4. Cytokine concentrations were normalized.

(B) Graphical Spearman correlation matrix of bacterial abundances found in the 94 cervical swabs. The areas and colors of circles show the absolute value of the corresponding correlation coefficients. Only correlations with $p < 0.01$ are shown.

A



B

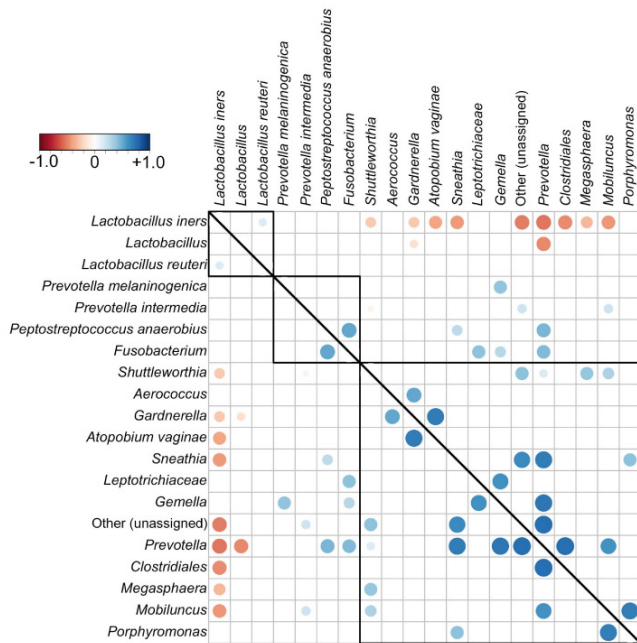


Figure A1.6 (Continued)

Table A1.1. Principal components analysis to reduce cytokine data dimensionality. Loading of each variable to the first principal component (explaining 41% of variation) and the second principal component (explaining 13% of variation.)

	Principal Component	
	PC1	PC2
	(41% of variation explained)	(13% of variation explained)
Loading of each cytokine	TNF- α	0.303
	IL-1 β	-0.213
	IL-4	-0.114
	IFN- γ	-0.088
	IL-12p70	-0.202
	IL-10	-0.255
	IL-2	-0.061
	sCD40L	0.176
	MIP-1 β	0.243
	MIP-1 α	-0.005
	IL-1 α	0.053
	FLT-3L	0.312
	IFN- α 2	0.223
	RANTES	0.199
	IP-10	0.457
	IL-1RA	0.494

Table A1.2. Demographics, sexual behavior, and progesterone levels in women in the top and bottom quartile of cytokine PC1. Women in the top quartile of cytokine PC1 had older current partners (1.91 year difference in means, $P = 0.0103$). Data shown as median with interquartile range or mean with standard deviation.

	Low PC1	High PC1	Significance
Age of sexual debut	18 [17, 19]	18 [17, 19]	$p = 0.9879^a$
Age of first sexual partner	21 [19, 22]	21 [20, 22.5]	$p = 0.2986^a$
Age of oldest sexual partner	26 [23, 27]	26 [24, 28]	$p = 0.4199^b$
Age of current sexual partner	23 [21, 26]	26 [23.5, 28]	$p = 0.0103^b$
Days since last sex episode	13 [4, 42]	12 [5.75, 42]	$p = 0.9528^b$
Number of sex acts in last 7 days	0 [0, 1]	0 [0, 1]	$p = 0.8735^b$
Number of sex acts in last 30 days	2 [0, 4]	2 [0, 4]	$p = 0.8619^b$
Blood progesterone level (ng/ml)	0.15 [0.1, 0.5]	0.1 [0.1, 0.2]	$p = 0.4008^b$
	Low PC1	High PC1	Significance ^c
No sex partners in last 7 days	16	12	$p = 0.2167$
1 or more sex partners	15	22	
No casual sex partners in last 7 days	31	34	$p = 1$
1 or more casual sex partners	0	0	
No oral sex	28	31	$p = 1$
Oral sex reported	2	2	
Always used condoms in past 30 days, or no sex	9	12	$p = 0.553$
Sometimes used condoms	12	11	
Never used condoms	6	11	
No family planning	19	15	$p = 0.2162$
Using family planning	12	19	
No STI history	27	23	$p = 1$
STI history	7	7	
No STI symptoms	30	32	$p = 1$
STI symptoms	1	2	

^a Unpaired T test

^b Mann-Whitney Test

^c Fisher's exact test

Table A1.3. Metadata from thirteen participants with longitudinal 16S profiling and cervicovaginal lavage measurements. Abbreviations: Cervicotype (CT), oral contraceptive pill (OCP).

	Days from baseline	CT	STI PCR	BV	# Sex episodes in 7 days	Days since last sex episode	# anal sex episodes in last 30 days	Condom use in last 30 days	History of STIs (self-reported)	STI symptoms now (self-reported)	Family planning (FP) method
Participant 12	0	4	-----	6	0	14	0	Always	No	No	IUD
Participant 12	98	4	-----	n/a	0	16	0	Always	No	Yes	IUD
Participant 12	186	4	-----	n/a	1	1	0	Always	No	No	IUD
Participant 12	249	4	NG+	7	1	2	0	Never	No	No	IUD
Participant 13	0	3	-----	0	2	5	0	Sometimes	No	No	Depoprovera
Participant 13	87	2	-----	1	2	7	0	Sometimes	No	No	Depoprovera
Participant 13	178	2	-----	n/a	2	3	0	Sometimes	No	No	Depoprovera
Participant 13	269	2	-----	1	n/a	n/a	n/a	n/a	n/a	n/a	Depoprovera
Participant 13	335	2	-----	0	0	12	0	Sometimes	No	No	Depoprovera
Participant 14	0	2	-----	0	1	7	0	Sometimes	No	No	No FP
Participant 14	81	2	n/a	n/a	3	4	0	Never	No	No	No FP
Participant 14	179	4	-----	n/a	1	3	0	Never	No	No	No FP
Participant 14	227	4	NG+	1	1	6	0	Never	No	No	No FP
Participant 15	0	1	-----	0	1	7	0	Never	No	No	Nuristerate
Participant 15	168	2	-----	n/a	1	4	0	Never	No	No	Nuristerate
Participant 15	245	2	-----	1	4	3	10	Never	No	No	Nuristerate
Participant 15	329	2	-----	1	4	2	0	Sometimes	Yes	No	Nuristerate
Participant 16	0	4	-----	9	0	60	0	Sometimes	No	No	No FP
Participant 16	171	4	-----	n/a	0	53	0	n/a	No	No	No FP
Participant 16	245	4	-----	6	0	26	0	Always	No	No	No FP
Participant 16	329	4	-----	n/a	0	8	0	Never	No	No	No FP
Participant 17	0	n/a	TV+	3	1	6	0	Always	No	No	OCP
Participant 17	88	4	n/a	n/a	1	7	0	Sometimes	No	No	OCP
Participant 17	179	4	HSV2 +	9	1	4	0	Never	No	No	OCP
Participant 17	227	4	n/a	n/a	5	2	0	Sometimes	No	No	OCP
Participant 18	0	2	-----	1	0	15	0	Sometimes	No	No	No FP
Participant 18	81	4	n/a	n/a	1	6	0	Sometimes	No	No	OCP
Participant 18	258	3	CT+	1	0	45	n/a	n/a	No	No	OCP
Participant 19	0	3	-----	6	2	3	0	Sometimes	No	No	Nuristerate
Participant 19	171	3	-----	n/a	0	17	1	Never	No	No	No FP
Participant 19	259	4	-----	9	0	34	n/a	n/a	n/a	No	No FP
Participant 19	337	4	-----	n/a	0	17	0	Sometimes	No	No	No FP
Participant 20	0	1	-----	0	1	7	0	Sometimes	No	No	No FP
Participant 20	172	2	-----	1	0	22	0	Sometimes	No	No	No FP
Participant 20	266	3	-----	1	0	18	0	Sometimes	No	No	No FP
Participant 21	0	2	-----	0	1	6	0	Sometimes	Yes	No	Depoprovera
Participant 21	81	4	-----	3	1	4	0	Sometimes	No	No	No FP
Participant 21	186	3	-----	n/a	1	4	0	Never	No	No	No FP
Participant 21	263	4	-----	9	0	30	0	Never	No	No	No FP
Participant 21	333	4	NG+	6	0	16	0	Sometimes	No	No	No FP
Participant 22	0	1	-----	1	1	6	0	Never	No	No	No FP
Participant 22	98	2	-----	n/a	1	5	0	Never	No	No	No FP
Participant 22	182	1	-----	n/a	3	1	0	Sometimes	No	No	No FP
Participant 22	241	1	NG+, CT+	2	1	3	0	Never	No	No	Nuristerate
Participant 23	0	2	-----	2	0	30	0	Never	Yes	No	Depoprovera
Participant 23	84	1	n/a	n/a	0	60	0	n/a	No	No	Depoprovera
Participant 23	171	2	-----	n/a	0	30	0	Sometimes	No	No	Depoprovera
Participant 23	230	2	-----	1	0	60	n/a	n/a	No	No	Depoprovera
Participant 24	0	4	-----	8	1	3	5	Never	No	No	No FP
Participant 24	94	4	-----	n/a	0	27	0	Sometimes	No	No	No FP
Participant 24	182	4	-----	n/a	0	9	0	Never	No	No	No FP
Participant 24	240	4	-----	6	1	4	1	Sometimes	No	Yes	No FP

Appendix 2

Corresponding to Chapter 3

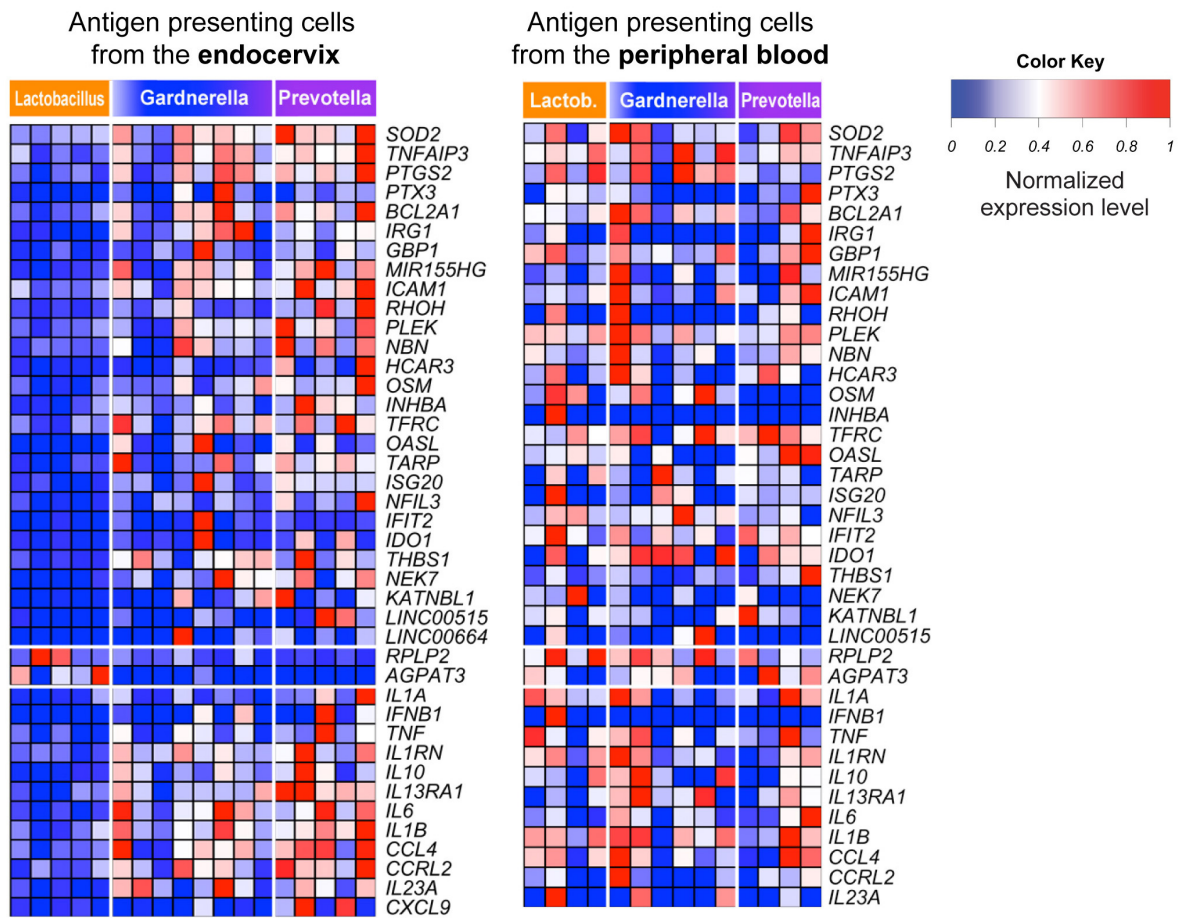


Figure A2.1 Expanded heatmap from Figure 3.2 displaying *Gardnerella* dominant communities as well. No difference in peripheral blood antigen presenting cells from women with different genital bacterial communities. The heatmap displays only genes that were significantly differentially expressed in cervical antigen presenting cells.

Table A2.1. Spearman correlations between individual bacteria and cytokine PC1.

Parameter	Spearman r	95% confidence interval	P value (two- tailed)
<i>Prevotella</i>	0.4443	0.2588 to 0.5982	< 0.0001
<i>Mycoplasma</i>	0.4781	0.2985 to 0.6251	< 0.0001
Other (unassigned)	0.5134	0.3404 to 0.6528	< 0.0001
<i>Gemella</i>	0.5234	0.3525 to 0.6606	< 0.0001
<i>Fusobacterium</i>	0.3882	0.1944 to 0.5528	0.0001
<i>Leptotrichiaceae</i>	0.3911	0.1977 to 0.5552	0.0001
<i>Lactobacillus iners</i>	-0.3585	-0.5284 to -0.1610	0.0004
<i>Sneathia</i>	0.3504	0.1520 to 0.5217	0.0006
<i>Aerococcus</i>	0.3445	0.1454 to 0.5168	0.0007
<i>Clostridiales</i>	0.3422	0.1429 to 0.5149	0.0008
<i>Mobiluncus</i>	0.3347	0.1345 to 0.5086	0.001
<i>Lactobacillus (non-iners)</i>	-0.2918	-0.4725 to -0.08760	0.0045
<i>Arcanobacterium</i>	0.2628	0.05625 to 0.4477	0.0109
<i>Atopobium vaginae</i>	0.2471	0.03951 to 0.4342	0.017
<i>Veillonella</i>	0.2279	0.01917 to 0.4175	0.028
<i>Porphyromonas</i>	0.1957	-0.01449 to 0.3893	0.0601
<i>Peptostreptococcus anaerobius</i>	0.1733	-0.03764 to 0.3695	0.0966
<i>Lactobacillus reuteri</i>	-0.1455	-0.3446 to 0.06609	0.164
<i>Prevotella intermedia</i>	0.143	-0.06868 to 0.3423	0.1715
<i>Gardnerella</i>	0.107	-0.1049 to 0.3097	0.3071
<i>Prevotella melaninogenica</i>	0.1022	-0.1097 to 0.3053	0.3296
<i>Shuttleworthia</i>	0.08394	-0.1279 to 0.2885	0.4238
<i>Megasphaera</i>	0.07931	-0.1325 to 0.2842	0.4498
<i>Prevotella copri</i>	-0.05216	-0.2589 to 0.1592	0.6195

Table A2.2. Bacterial pathways identified by HUMAnN to be differentially abundant between women with high versus low genital inflammation.

KEGG Pathway	Enriched in:	LDA Score (Log 10)
ko00780 Biotin metabolism	HighInflamm	3.290
ko00540 Lipopolysaccharide biosynthesis	HighInflamm	3.201
ko00750 Vitamin B6 metabolism	HighInflamm	3.157
ko00410 beta-Alanine metabolism	HighInflamm	3.149
ko02040 Flagellar assembly	HighInflamm	3.110
ko02030 Bacterial chemotaxis	HighInflamm	3.088
ko00523 Polyketide sugar unit biosynthesis	HighInflamm	3.042
ko05310 Asthma	HighInflamm	3.042
ko00660 C5-Branched dibasic acid metabolism	HighInflamm	2.986
ko00860 Porphyrin and chlorophyll metabolism	HighInflamm	2.915
ko00340 Histidine metabolism	HighInflamm	2.885
ko00260 Glycine, serine and threonine metabolism	HighInflamm	2.871
ko00361 Chlorocyclohexane and chlorobenzene degradation	HighInflamm	2.850
ko00330 Arginine and proline metabolism	HighInflamm	2.715
ko05131 Shigellosis	HighInflamm	2.525
ko00562 Inositol phosphate metabolism	HighInflamm	2.490
ko05120 Epithelial cell signaling in Helicobacter pylori infection	HighInflamm	2.311
ko02060 Phosphotransferase system (PTS)	LowInflammation	3.503
ko03430 Mismatch repair	LowInflammation	3.227
ko00240 Pyrimidine metabolism	LowInflammation	3.221
ko00051 Fructose and mannose metabolism	LowInflammation	3.215
ko00072 Synthesis and degradation of ketone bodies	LowInflammation	3.112
ko00052 Galactose metabolism	LowInflammation	3.100
ko00520 Amino sugar and nucleotide sugar metabolism	LowInflammation	3.060
ko00230 Purine metabolism	LowInflammation	2.962
ko00010 Glycolysis / Gluconeogenesis	LowInflammation	2.903
ko00561 Glycerolipid metabolism	LowInflammation	2.865
ko03020 RNA polymerase	LowInflammation	2.794
ko00621 Dioxin degradation	LowInflammation	2.728
ko05150 Staphylococcus aureus infection	LowInflammation	2.640
ko02010 ABC transporters	LowInflammation	2.603
ko04662 B cell receptor signaling pathway	LowInflammation	2.331

Appendix 3

Corresponding to Chapter 4

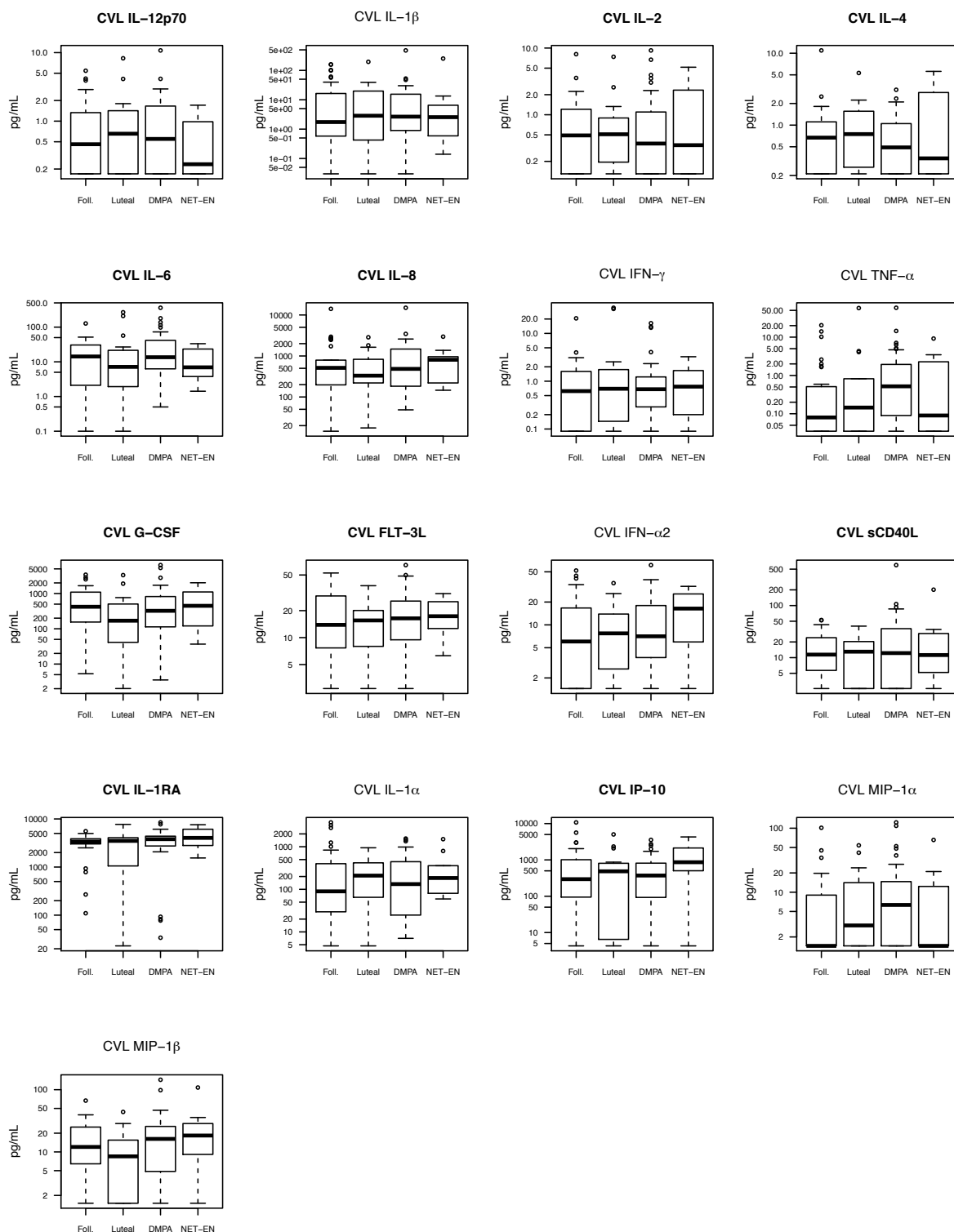


Figure A3.1 Concentrations of cytokines in cervicovaginal lavages among naturally cycling women in the follicular phase (n=33) or luteal phase (n=16) of the menstrual cycle, or women using DMPA (n=41) or NET-EN (n=10).